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(54) Title: ARRAYS OF NUCLEIC ACID PROBES ON BIOLOGICAL CHIPS (57) Abstract <p>The invention provides chips of immobilized probes, and methods employing the chips, for comparing a reference polynucleotide sequence of known sequence with a target sequence showing substantial similarity with the reference sequence, but differing in the presence of e.g., mutations.</p>		

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ARRAYS OF NUCLEIC ACID PROBES ON BIOLOGICAL CHIPS5 Cross-Reference to Related Application

This application is a continuation-in-part of USSN 08/284,064, filed August 2, 1994, which is a continuation-in-part of USSN 08/143,312, filed October 26, 1993, each of which is incorporated by reference in its entirety for all purposes. 10 Research leading to the invention was funded in part by NIH grant No. 1R01HG00813-01, and the government may have certain rights to the invention.

Background of the Invention15 Field of the Invention

The present invention provides arrays of oligonucleotide probes immobilized in microfabricated patterns on silica chips for analyzing molecular interactions of biological interest. The invention therefore relates to diverse fields impacted by 20 the nature of molecular interaction, including chemistry, biology, medicine, and medical diagnostics.

Description of Related Art

Oligonucleotide probes have long been used to detect 25 complementary nucleic acid sequences in a nucleic acid of interest (the "target" nucleic acid). In some assay formats, the oligonucleotide probe is tethered, *i.e.*, by covalent attachment, to a solid support, and arrays of oligonucleotide probes immobilized on solid supports have been used to detect 30 specific nucleic acid sequences in a target nucleic acid. See, *e.g.*, PCT patent publication Nos. WO 89/10977 and 89/11548. Others have proposed the use of large numbers of oligonucleotide probes to provide the complete nucleic acid sequence of a target nucleic acid but failed to provide an 35 enabling method for using arrays of immobilized probes for this purpose. See U.S. Patent Nos. 5,202,231 and 5,002,867 and PCT patent publication No. WO 93/17126.

The development of VLSIPS™ technology has provided methods for making very large arrays of oligonucleotide probes in very small arrays. See U.S. Patent No. 5,143,854 and PCT patent publication Nos. WO 90/15070 and 92/10092, each of which is incorporated herein by reference. U.S. Patent application Serial No. 082,937, filed June 25, 1993, describes methods for making arrays of oligonucleotide probes that can be used to provide the complete sequence of a target nucleic acid and to detect the presence of a nucleic acid containing a specific nucleotide sequence.

Microfabricated arrays of large numbers of oligonucleotide probes, called "DNA chips" offer great promise for a wide variety of applications. New methods and reagents are required to realize this promise, and the present invention helps meet that need.

SUMMARY OF THE INVENTION

The invention provides several strategies employing immobilized arrays of probes for comparing a reference sequence of known sequence with a target sequence showing substantial similarity with the reference sequence, but differing in the presence of, e.g., mutations. In a first embodiment, the invention provides a tiling strategy employing an array of immobilized oligonucleotide probes comprising at least two sets of probes. A first probe set comprises a plurality of probes, each probe comprising a segment of at least three nucleotides exactly complementary to a subsequence of the reference sequence, the segment including at least one interrogation position complementary to a corresponding nucleotide in the reference sequence. A second probe set comprises a corresponding probe for each probe in the first probe set, the corresponding probe in the second probe set being identical to a sequence comprising the corresponding probe from the first probe set or a subsequence of at least three nucleotides thereof that includes the at least one interrogation position, except that the at least one interrogation position is occupied by a different nucleotide in each of the two corresponding probes from the first and second probe sets. The probes in the first probe set have at

least two interrogation positions corresponding to two contiguous nucleotides in the reference sequence. One interrogation position corresponds to one of the contiguous nucleotides, and the other interrogation position to the other.

In a second embodiment, the invention provides a tiling strategy employing an array comprising four probe sets. A first probe set comprises a plurality of probes, each probe comprising a segment of at least three nucleotides exactly complementary to a subsequence of the reference sequence, the segment including at least one interrogation position complementary to a corresponding nucleotide in the reference sequence. Second, third and fourth probe sets each comprise a corresponding probe for each probe in the first probe set. The probes in the second, third and fourth probe sets are identical to a sequence comprising the corresponding probe from the first probe set or a subsequence of at least three nucleotides thereof that includes the at least one interrogation position, except that the at least one interrogation position is occupied by a different nucleotide in each of the four corresponding probes from the four probe sets. The first probe set often has at least 100 interrogation positions corresponding to 100 contiguous nucleotides in the reference sequence. Sometimes the first probe set has an interrogation position corresponding to every nucleotide in the reference sequence. The segment of complementarity within the probe set is usually about 9-21 nucleotides. Although probes may contain leading or trailing sequences in addition to the 9-21 sequences, many probes consist exclusively of a 9-21 segment of complementarity.

In a third embodiment, the invention provides immobilized arrays of probes tiled for multiple reference sequences. One such array comprises at least one pair of first and second probe groups, each group comprising first and second sets of probes as defined in the first embodiment. Each probe in the first probe set from the first group is exactly complementary to a subsequence of a first reference sequence, and each probe in the first probe set from the second group is exactly

complementary to a subsequence of a second reference sequence. Thus, the first group of probes are tiled with respect to a first reference sequence and the second group of probes with respect to a second reference sequence. Each group of probes
5 can also include third and fourth sets of probes as defined in the second embodiment. In some arrays of this type, the second reference sequence is a mutated form of the first reference sequence.

In a fourth embodiment, the invention provides arrays for
10 block tiling. Block tiling is a species of the general tiling strategies described above. The usual unit of a block tiling array is a group of probes comprising a wildtype probe, a first set of three mutant probes and a second set of three mutant probes. The wildtype probe comprises a segment of at
15 least three nucleotides exactly complementary to a subsequence of a reference sequence. The segment has at least first and second interrogation positions corresponding to first and second nucleotides in the reference sequence. The probes in the first set of three mutant probes are each identical to a
20 sequence comprising the wildtype probe or a subsequence of at least three nucleotides thereof including the first and second interrogation positions, except in the first interrogation position, which is occupied by a different nucleotide in each of the three mutant probes and the wildtype probe. The probes
25 in the second set of three mutant probes are each identical to a sequence comprising the wildtype probes or a subsequence of at least three nucleotides thereof including the first and second interrogation positions, except in the second interrogation position, which is occupied by a different
30 nucleotide in each of the three mutant probes and the wildtype probe.

In a fifth embodiment, the invention provides methods of comparing a target sequence with a reference sequence using
arrays of immobilized pooled probes. The arrays employed in
35 these methods represent a further species of the general tiling arrays noted above. In these methods, variants of a reference sequence differing from the reference sequence in at least one nucleotide are identified and each is assigned a

designation. An array of pooled probes is provided, with each pool occupying a separate cell of the array. Each pool comprises a probe comprising a segment exactly complementary to each variant sequence assigned a particular designation.

5 The array is then contacted with a target sequence comprising a variant of the reference sequence. The relative hybridization intensities of the pools in the array to the target sequence are determined. The identity of the target sequence is deduced from the pattern of hybridization

10 intensities. Often, each variant is assigned a designation having at least one digit and at least one value for the digit. In this case, each pool comprises a probe comprising a segment exactly complementary to each variant sequence assigned a particular value in a particular digit. When

15 variants are assigned successive numbers in a numbering system of base m having n digits, $n \times (m-1)$ pooled probes are used are used to assign each variant a designation.

In a sixth embodiment, the invention provides a pooled probe for trellis tiling, a further species of the general

20 tiling strategy. In trellis tiling, the identity of a nucleotide in a target sequence is determined from a comparison of hybridization intensities of three pooled trellis probes. A pooled trellis probe comprises a segment exactly complementary to a subsequence of a reference sequence

25 except at a first interrogation position occupied by a pooled nucleotide N , a second interrogation position occupied by a pooled nucleotide selected from the group of three consisting of (1) M or K , (2) R or Y and (3) S or W , and a third interrogation position occupied by a second pooled nucleotide

30 selected from the group. The pooled nucleotide occupying the second interrogation position comprises a nucleotide complementary to a corresponding nucleotide from the reference sequence when the second pooled probe and reference sequence are maximally aligned, and the pooled nucleotide occupying the

35 third interrogation position comprises a nucleotide complementary to a corresponding nucleotide from the reference sequence when the third pooled probe and the reference

sequence are maximally aligned. Standard IUPAC nomenclature is used for describing pooled nucleotides.

In trellis tiling, an array comprises at least first, second and third cells, respectively occupied by first, second and third pooled probes, each according to the generic description above. However, the segment of complementarity, location of interrogation positions, and selection of pooled nucleotide at each interrogation position may or may not differ between the three pooled probes subject to the following constraint. One of the three interrogation positions in each of the three pooled probes must align with the same corresponding nucleotide in the reference sequence. This interrogation position must be occupied by a N in one of the pooled probes, and a different pooled nucleotide in each of the other two pooled probes.

In a seventh embodiment, the invention provides arrays for bridge tiling. Bridge tiling is a species of the general tiling strategies noted above, in which probes from the first probe set contain more than one segment of complementarity. In bridge tiling, a nucleotide in a reference sequence is usually determined from a comparison of four probes. A first probe comprises at least first and second segments, each of at least three nucleotides and each exactly complementary to first and second subsequences of a reference sequences. The segments including at least one interrogation position corresponding to a nucleotide in the reference sequence. Either (1) the first and second subsequences are noncontiguous in the reference sequence, or (2) the first and second subsequences are contiguous and the first and second segments are inverted relative to the first and second subsequences. The arrays further comprises second, third and fourth probes, which are identical to a sequence comprising the first probe or a subsequence thereof comprising at least three nucleotides from each of the first and second segments, except in the at least one interrogation position, which differs in each of the probes. In a species of bridge tiling, referred to as deletion tiling, the first and second subsequences are separated by one or two nucleotides in the reference sequence.

In an eighth embodiment, the invention provides arrays of probes for multiplex tiling. Multiplex tiling is a strategy, in which the identity of two nucleotides in a target sequence is determined from a comparison of the hybridization intensities of four probes, each having two interrogation positions. Each of the probes comprising a segment of at least 7 nucleotides that is exactly complementary to a subsequence from a reference sequence, except that the segment may or may not be exactly complementary at two interrogation positions. The nucleotides occupying the interrogation positions are selected by the following rules: (1) the first interrogation position is occupied by a different nucleotide in each of the four probes, (2) the second interrogation position is occupied by a different nucleotide in each of the four probes, (3) in first and second probes, the segment is exactly complementary to the subsequence, except at no more than one of the interrogation positions, (4) in third and fourth probes, the segment is exactly complementary to the subsequence, except at both of the interrogation positions.

In a ninth embodiment, the invention provides arrays of immobilized probes including helper mutations. Helper mutations are useful for, e.g., preventing self-annealing of probes having inverted repeats. In this strategy, the identity of a nucleotide in a target sequence is usually determined from a comparison of four probes. A first probe comprises a segment of at least 7 nucleotides exactly complementary to a subsequence of a reference sequence except at one or two positions, the segment including an interrogation position not at the one or two positions. The one or two positions are occupied by helper mutations. Second, third and fourth mutant probes are each identical to a sequence comprising the wildtype probe or a subsequence thereof including the interrogation position and the one or two positions, except in the interrogation position, which is occupied by a different nucleotide in each of the four probes.

In a tenth embodiment, the invention provides arrays of probes comprising at least two probe sets, but lacking a probe set comprising probes that are perfectly matched to a

reference sequence. Such arrays are usually employed in methods in which both reference and target sequence are hybridized to the array. The first probe set comprising a plurality of probes, each probe comprising a segment exactly complementary to a subsequence of at least 3 nucleotides of a reference sequence except at an interrogation position. The second probe set comprises a corresponding probe for each probe in the first probe set, the corresponding probe in the second probe set being identical to a sequence comprising the corresponding probe from the first probe set or a subsequence of at least three nucleotides thereof that includes the interrogation position, except that the interrogation position is occupied by a different nucleotide in each of the two corresponding probes and the complement to the reference sequence.

In an eleventh embodiment, the invention provides methods of comparing a target sequence with a reference sequence comprising a predetermined sequence of nucleotides using any of the arrays described above. The methods comprise hybridizing the target nucleic acid to an array and determining which probes, relative to one another, in the array bind specifically to the target nucleic acid. The relative specific binding of the probes indicates whether the target sequence is the same or different from the reference sequence. In some such methods, the target sequence has a substituted nucleotide relative to the reference sequence in at least one undetermined position, and the relative specific binding of the probes indicates the location of the position and the nucleotide occupying the position in the target sequence. In some methods, a second target nucleic acid is also hybridized to the array. The relative specific binding of the probes then indicates both whether the target sequence is the same or different from the reference sequence, and whether the second target sequence is the same or different from the reference sequence. In some methods, when the array comprises two groups of probes tiled for first and second reference sequences, respectively, the relative specific binding of probes in the first group indicates whether the

target sequence is the same or different from the first reference sequence. The relative specific binding of probes in the second group indicates whether the target sequence is the same or different from the second reference sequence.

5 Such methods are particularly useful for analyzing heterologous alleles of a gene. Some methods entail hybridizing both a reference sequence and a target sequence to any of the arrays of probes described above. Comparison of the relative specific binding of the probes to the reference
10 and target sequences indicates whether the target sequence is the same or different from the reference sequence.

In a twelfth embodiment, the invention provides arrays of immobilized probes in which the probes are designed to tile a reference sequence from a human immunodeficiency virus.
15 Reference sequences from either the reverse transcriptase gene or protease gene of HIV are of particular interest. Some chips further comprise arrays of probes tiling a reference sequence from a 16S RNA or DNA encoding the 16S RNA from a pathogenic microorganism. The invention further provides
20 methods of using such arrays in analyzing a HIV target sequence. The methods are particularly useful where the target sequence has a substituted nucleotide relative to the reference sequence in at least one position, the substitution conferring resistance to a drug use in treating a patient
25 infected with a HIV virus. The methods reveal the existence of the substituted nucleotide. The methods are also particularly useful for analyzing a mixture of undetermined proportions of first and second target sequences from different HIV variants. The relative specific binding of
30 probes indicates the proportions of the first and second target sequences.

In a thirteenth embodiment, the invention provides arrays of probes tiled based on reference sequence from a CFTR gene. A preferred array comprises at least a group of probes
35 comprising a wildtype probe, and five sets of three mutant probes. The wildtype probe is exactly complementary to a subsequence of a reference sequence from a cystic fibrosis gene, the segment having at least five interrogation positions

corresponding to five contiguous nucleotides in the reference sequence. The probes in the first set of three mutant probes are each identical to the wildtype probe, except in a first of the five interrogation positions, which is occupied by a
5 different nucleotide in each of the three mutant probes and the wildtype probe. The probes in the second set of three mutant probes are each identical to the wildtype probe, except in a second of the five interrogation positions, which is occupied by a different nucleotide in each of the three mutant
10 probes and the wildtype probe. The probes in the third set of three mutant probes are each identical to the wildtype probe, except in a third of the five interrogation positions, which is occupied by a different nucleotide in each of the three mutant probes and the wildtype probe. The probes in the
15 fourth set of three mutant probes are each identical to the wildtype probe, except in a fourth of the five interrogation positions, which is occupied by a different nucleotide in each of the three mutant probes and the wildtype probe. The probes in the fifth set of three mutant probes are each identical to
20 the wildtype probe, except in a fifth of the five interrogation positions, which is occupied by a different nucleotide in each of the three mutant probes and the wildtype probe. Preferably, a chip comprises two such groups of probes. The first group comprises a wildtype probe exactly
25 complementary to a first reference sequence, and the second group comprises a wildtype probe exactly complementary to a second reference sequence that is a mutated form of the first reference sequence.

The invention further provides methods of using the
30 arrays of the invention for analyzing target sequences from a CFTR gene. The methods are capable of simultaneously analyzing first and second target sequences representing heterozygous alleles of a CFTR gene.

In a fourteenth embodiment, the invention provides arrays
35 of probes tiling a reference sequence from a p53 gene, an hMLH1 gene and/or an MSH2 gene. The invention further provides methods of using the arrays described above to

analyze these genes. The methods are useful, e.g., for diagnosing patients susceptible to developing cancer.

In a fifteenth embodiment, the invention provides arrays of probes tiling a reference sequence from a mitochondrial genome. The reference sequence may comprise part or all of the D-loop region, or all, or substantially all, of the mitochondrial genome. The invention further provides method of using the arrays described above to analyze target sequences from a mitochondrial genome. The methods are useful for identifying mutations associated with disease, and for forensic, epidemiological and evolutionary studies.

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1: Basic tiling strategy. The figure illustrates the relationship between an interrogation position (I) and a corresponding nucleotide (n) in the reference sequence, and between a probe from the first probe set and corresponding probes from second, third and fourth probe sets.

Fig. 2: Segment of complementarity in a probe from the first probe set.

Fig. 3: Incremental succession of probes in a basic tiling strategy. The figure shows four probe sets, each having three probes. Note that each probe differs from its predecessor in the same set by the acquisition of a 5' nucleotide and the loss of a 3' nucleotide, as well as in the nucleotide occupying the interrogation position.

Fig. 4: Exemplary arrangement of lanes on a chip. The chip shows four probe sets, each having five probes and each having a total of five interrogation positions (I1-I5), one per probe.

Fig. 5: Hybridization pattern of chip having probes laid down in lanes. Dark patches indicate hybridization. The probes in the lower part of the figure occur at the column of the array indicated by the arrow when the probes length is 15 and the interrogation position 7.

Fig. 6: Strategies for detecting deletion and insertion mutations. Bases in brackets may or may not be present.

Fig. 7: Block tiling strategy. The probe from the first probe set has three interrogation positions. The probes from the other probe sets have only one of these interrogation positions.

5 Fig. 8: Multiplex tiling strategy. Each probe has two interrogation positions.

Fig. 9. Helper mutation strategy. The segment of complementarity differs from the complement of the reference sequence at a helper mutation as well as the interrogation
10 position.

Fig. 10 Layout of probes on the HV 407 chip. The figure shows successive rows of sequence each of which is subdivided into four lanes. The four lanes correspond to the A-, C-, G- and T-lanes on the chip. Each probe is represented by the
15 nucleotide occupying its interrogation position. The letter "N" indicates a control probe or empty column. The different sized-probes are laid out in parallel. That is, from top-to-bottom, a row of 13 mers is followed by a row of 15 mers, which is followed by a row of 17 mers, which is followed by a
20 row of 19 mers.

Fig. 11 Fluorescence pattern of HV 407 hybridized to a target sequence (pPol19) identical to the chips reference sequence.

Fig. 12 Sequence read from HV 407 chip hybridized to
25 pPol19 and 4MUT18 (separate experiments). The reference sequence is designated "wildtype." Beneath the reference sequence are four rows of sequence read from the chip hybridized to the pPol19 target, the first row being read from 13 mers, the second row from 15 mers, the third row from 17
30 mers and the fourth row from 19 mers. Beneath these sequences, there are four further rows of sequence read from the chip hybridized to the HXB2 target. Successive rows are read from 13 mers, 15 mers, 17 mers and 19 mers. Each
35 nucleotide in a row is called from the relative fluorescence intensities of probes in A-, C-, G- and T-lanes. Regions of ambiguous sequence read from the chip are highlighted. The strain differences between the HXB2 sequence and the reference sequence that were correctly detected are indicated (*), and

those that could not be called are indicated (o). (The nucleotide at position 417 was read correctly in some experiments). The location of some mutations known to be associated with drug resistance that occur in readable regions of the chip are shown above (codon number) and below (mutant nucleotide) the sequence designated "wildtype." The locations of primer used to amplify the target sequence are indicated by arrows.

Fig. 13: Detection of mixed target sequences. The mutant target differs from the wildtype by a single mutation in codon 67 of the reverse transcriptase gene. Each different sized group of probes has a column of four probes for reading the nucleotide in which the mutation occurs. The four probes occupying a column are represented by a single probe in the figure with the symbol (o) indicating the interrogation position, which is occupied by a different nucleotide in each probe.

Fig. 14: Fluorescence intensities of target bound to 13 mers and 15 mers for different proportions of mutant and wildtype target. The fluorescence intensities are from probes having interrogation positions for reading the nucleotide at which the mutant and wildtype targets diverge.

Fig. 15: Sequence read from protease chip from four clinical samples before and after treatment with ddI>.

Fig. 16: Block tiling array of probes for analyzing a CFTR point mutation. Each probe show actually represents four probes, with one probe having each of A, C, G or T at the interrogation position N. In the order shown, the first probe shown on the left is tiled from the wildtype reference sequence, the second probe from the mutant sequence, and so on in alternating fashion. Note that all of the probes are identical except at the interrogation position, which shifts one position between successive probes tiled from the same reference sequence (e.g., the first, third and fifth probes in the left hand column.) The grid shows the hybridization intensities when the array is hybridized to the reference sequence.

Fig. 17: Hybridization pattern for heterozygous target. The figure shows the hybridization pattern when the array of the previous figure is hybridized to a mixture of mutant and wildtype reference sequences.

5 Fig. 18, in panels A, B, and C, shows an image made from the region of a DNA chip containing CFTR exon 10 probes; in panel A, the chip was hybridized to a wild-type target; in panel C, the chip was hybridized to a mutant $\Delta F508$ target; and in panel B, the chip was hybridized to a mixture of the
10 wild-type and mutant targets.

 Fig. 19, in sheets 1 - 3, corresponding to panels A, B, and C of Fig. 18, shows graphs of fluorescence intensity versus tiling position. The labels on the horizontal axis show the bases in the wild-type sequence corresponding to the
15 position of substitution in the respective probes. Plotted are the intensities observed from the features (or synthesis sites) containing wild-type probes, the features containing the substitution probes that bound the most target ("called"), and the feature containing the substitution probes that bound
20 the target with the second highest intensity of all the substitution probes ("2nd Highest").

 Fig. 20, in panels A, B, and C, shows an image made from a region of a DNA chip containing CFTR exon 10 probes; in panel A, the chip was hybridized to the wt480 target; in panel
25 C, the chip was hybridized to the mu480 target; and in panel B, the chip was hybridized to a mixture of the wild-type and mutant targets.

 Fig. 21, in sheets 1 - 3, corresponding to panels A, B, and C of Fig. 20, shows graphs of fluorescence intensity
30 versus tiling position. The labels on the horizontal axis show the bases in the wild-type sequence corresponding to the position of substitution in the respective probes. Plotted are the intensities observed from the features (or synthesis sites) containing wild-type probes, the features containing
35 the substitution probes that bound the most target ("called"), and the feature containing the substitution probes that bound the target with the second highest intensity of all the substitution probes ("2nd Highest").

Fig. 22, in panels A and B, shows an image made from a region of a DNA chip containing CFTR exon 10 probes; in panel A, the chip was hybridized to nucleic acid derived from the genomic DNA of an individual with wild-type $\Delta F508$ sequences; in panel B, the target nucleic acid originated from a heterozygous (with respect to the $\Delta F508$ mutation) individual.

Fig. 23, in sheets 1 and 2, corresponding to panels A and B of Fig. 22, shows graphs of fluorescence intensity versus tiling position. The labels on the horizontal axis show the bases in the wild-type sequence corresponding to the position of substitution in the respective probes. Plotted are the intensities observed from the features (or synthesis sites) containing wild-type probes, the features containing the substitution probes that bound the most target ("called"), and the feature containing the substitution probes that bound the target with the second highest intensity of all the substitution probes ("2nd Highest").

Fig. 24: Hybridization of homozygous wildtype (A) and heterozygous (B) target sequences from exon 11 of the CFTR gene to a block tiling array designed to detect G551D and Q552X mutations in CFTR gene.

Fig. 25: Hybridization of homozygous wildtype (A) and $\Delta F508$ mutant (B) target sequences from exon 10 of the CFTR gene to a block tiling array designed to detect mutations, $\Delta F508$, $\Delta I507$ and F508C.

Fig. 26: Hybridization of heterozygous mutant target sequences, $\Delta F508/F508C$, to the array of Fig. 25.

Fig. 27 shows the alignment of some of the probes on a p53 DNA chip with a 12-mer model target nucleic acid.

Fig. 28 shows a set of 10-mer probes for a p53 exon 6 DNA chip.

Fig. 29 shows that very distinct patterns are observed after hybridization of p53 DNA chips with targets having different 1 base substitutions. In the first image in Fig. 29, the 12-mer probes that form perfect matches with the wild-type target are in the first row (top). The 12-mer probes with single base mismatches are located in the second, third, and fourth rows and have much lower signals.

Fig. 30, in graphs 2, 3, and 4, graphically depicts the data in Fig. 29. On each graph, the X ordinate is the position of the probe in its row on the chip, and the Y ordinate is the signal at that probe site after hybridization.

5 Fig. 31 shows the results of hybridizing mixed target populations of WT and mutant p53 genes to the p53 DNA chip.

Fig. 32, in graphs 1-4, shows (see Fig. 30 as well) the hybridization efficiency of a 10-mer probe array as compared to a 12-mer probe array.

10 Fig. 33 shows an image of a p53 DNA chip hybridized to a target DNA.

Fig. 34 illustrates how the actual sequence was read from the chip shown in Fig. 33. Gaps in the sequence of letters in the WT rows correspond to control probes or sites. Positions at which bases are miscalled are represented by letters in italic type in cells corresponding to probes in which the WT bases have been substituted by other bases.

15 Fig. 35 shows the human mitochondrial genome; "O_H" is the H strand origin of replication, and arrows indicate the cloned unshaded sequence.

20 Fig. 36 shows the image observed from application of a sample of mitochondrial DNA derived nucleic acid (from the mt4 sample) on a DNA chip.

Fig. 37 is similar to Fig. 36 but shows the image observed from the mt5 sample.

25 Fig. 38 shows the predicted difference image between the mt4 and mt5 samples on the DNA chip based on mismatches between the two samples and the reference sequence.

Fig. 39 shows the actual difference image observed for the mt4 and mt5 samples.

30 Fig. 40, in sheets 1 and 2, shows a plot of normalized intensities across rows 10 and 11 of the array and a tabulation of the mutations detected.

Fig. 41 shows the discrimination between wild-type and mutant hybrids obtained with the chip. A median of the six normalized hybridization scores for each probe was taken; the graph plots the ratio of the median score to the normalized

hybridization score versus mean counts. A ratio of 1.6 and mean counts above 50 yield no false positives.

Fig. 42 illustrates how the identity of the base mismatch may influence the ability to discriminate mutant and wild-type sequences more than the position of the mismatch within an oligonucleotide probe. The mismatch position is expressed as % of probe length from the 3'-end. The base change is indicated on the graph.

Fig. 43 provides a 5' to 3' sequence listing of one target corresponding to the probes on the chip. X is a control probe. Positions that differ in the target (i.e., are mismatched with the probe at the designated site) are in bold.

Fig. 44 shows the fluorescence image produced by scanning the chip described in Fig. 17 when hybridized to a sample.

Fig. 45 illustrates the detection of 4 transitions in the target sequence relative to the wild-type probes on the chip in Fig. 44.

Fig. 46: VLSIPS™ technology applied to the light directed synthesis of oligonucleotides. Light (hv) is shone through a mask (M_1) to activate functional groups (-OH) on a surface by removal of a protecting group (X). Nucleoside building blocks protected with photoremovable protecting groups (T-X, C-X) are coupled to the activated areas. By repeating the irradiation and coupling steps, very complex arrays of oligonucleotides can be prepared.

Fig. 47: Use of the VLSIPS™ process to prepare "nucleoside combinatorials" or oligonucleotides synthesized by coupling all four nucleosides to form dimers, trimers, and so forth.

Fig. 48: Deprotection, coupling, and oxidation steps of a solid phase DNA synthesis method.

Fig. 49: An illustrative synthesis route for the nucleoside building blocks used in the VLSIPS™ method.

Fig. 50: A preferred photoremovable protecting group, MeNPOC, and preparation of the group in active form.

Fig. 51: Detection system for scanning a DNA chip.

DETAILED DESCRIPTION OF THE INVENTION

The invention provides a number of strategies for comparing a polynucleotide of known sequence (a reference sequence) with variants of that sequence (target sequences).

5 The comparison can be performed at the level of entire genomes, chromosomes, genes, exons or introns, or can focus on individual mutant sites and immediately adjacent bases. The strategies allow detection of variations, such as mutations or polymorphisms, in the target sequence irrespective whether a
10 particular variant has previously been characterized. The strategies both define the nature of a variant and identify its location in a target sequence.

The strategies employ arrays of oligonucleotide probes immobilized to a solid support. Target sequences are analyzed
15 by determining the extent of hybridization at particular probes in the array. The strategy in selection of probes facilitates distinction between perfectly matched probes and probes showing single-base or other degrees of mismatches. The strategy usually entails sampling each nucleotide of
20 interest in a target sequence several times, thereby achieving a high degree of confidence in its identity. This level of confidence is further increased by sampling of adjacent nucleotides in the target sequence to nucleotides of interest. The number of probes on the chip can be quite large (e.g.,
25 10^5 - 10^6). However, usually only a small proportion of the total number of probes of a given length are represented. Some advantage of the use of only a small proportion of all possible probes of a given length include: (i) each position in the array is highly informative, whether or not
30 hybridization occurs; (ii) nonspecific hybridization is minimized; (iii) it is straightforward to correlate hybridization differences with sequence differences, particularly with reference to the hybridization pattern of a known standard; and (iv) the ability to address each probe
35 independently during synthesis, using high resolution photolithography, allows the array to be designed and optimized for any sequence. For example the length of any probe can be varied independently of the others.

The present tiling strategies result in sequencing and comparison methods suitable for routine large-scale practice with a high degree of confidence in the sequence output.

5 I. GENERAL TILING STRATEGIES

A. Selection of Reference Sequence

The chips are designed to contain probes exhibiting complementarity to one or more selected reference sequence whose sequence is known. The chips are used to read a target
10 sequence comprising either the reference sequence itself or variants of that sequence. Target sequences may differ from the reference sequence at one or more positions but show a high overall degree of sequence identity with the reference sequence (e.g., at least 75, 90, 95, 99, 99.9 or 99.99%). Any
15 polynucleotide of known sequence can be selected as a reference sequence. Reference sequences of interest include sequences known to include mutations or polymorphisms associated with phenotypic changes having clinical significance in human patients. For example, the CFTR gene
20 and P53 gene in humans have been identified as the location of several mutations resulting in cystic fibrosis or cancer respectively. Other reference sequences of interest include those that serve to identify pathogenic microorganisms and/or are the site of mutations by which such microorganisms acquire
25 drug resistance (e.g., the HIV reverse transcriptase gene). Other reference sequences of interest include regions where polymorphic variations are known to occur (e.g., the D-loop region of mitochondrial DNA). These reference sequences have utility for, e.g., forensic or epidemiological studies. Other
30 reference sequences of interest include p34 (related to p53), p65 (implicated in breast, prostate and liver cancer), and DNA segments encoding cytochromes P450 (see Meyer et al., *Pharmac. Ther.* 46, 349-355 (1990)). Other reference sequences of
35 interest include those from the genome of pathogenic viruses (e.g., hepatitis (A, B, or C), herpes virus (e.g., VZV, HSV-1, HAV-6, HSV-II, and CMV, Epstein Barr virus), adenovirus, influenza virus, flaviviruses, echovirus, rhinovirus, coxsackie virus, cornovirus, respiratory syncytial virus,

mumps virus, rotavirus, measles virus, rubella virus, parvovirus, vaccinia virus, HTLV virus, dengue virus, papillomavirus, molluscum virus, poliovirus, rabies virus, JC virus and arboviral encephalitis virus. Other reference sequences of interest are from genomes or episomes of pathogenic bacteria, particularly regions that confer drug resistance or allow phylogenetic characterization of the host (e.g., 16S rRNA or corresponding DNA). For example, such bacteria include chlamydia, rickettsial bacteria, mycobacteria, staphylococci, streptococci, pneumococci, meningococci and gonococci, klebsiella, proteus, serratia, pseudomonas, legionella, diphtheria, salmonella, bacilli, cholera, tetanus, botulism, anthrax, plague, leptospirosis, and Lyme disease bacteria. Other reference sequences of interest include those in which mutations result in the following autosomal recessive disorders: sickle cell anemia, β -thalassemia, phenylketonuria, galactosemia, Wilson's disease, hemochromatosis, severe combined immunodeficiency, alpha-1-antitrypsin deficiency, albinism, alkaptonuria, lysosomal storage diseases and Ehlers-Danlos syndrome. Other reference sequences of interest include those in which mutations result in X-linked recessive disorders: hemophilia, glucose-6-phosphate dehydrogenase, agammaglobulinemia, diabetes insipidus, Lesch-Nyhan syndrome, muscular dystrophy, Wiskott-Aldrich syndrome, Fabry's disease and fragile X-syndrome. Other reference sequences of interest includes those in which mutations result in the following autosomal dominant disorders: familial hypercholesterolemia, polycystic kidney disease, Huntington's disease, hereditary spherocytosis, Marfan's syndrome, von Willebrand's disease, neurofibromatosis, tuberous sclerosis, hereditary hemorrhagic telangiectasia, familial colonic polyposis, Ehlers-Danlos syndrome, myotonic dystrophy, muscular dystrophy, osteogenesis imperfecta, acute intermittent porphyria, and von Hippel-Lindau disease.

The length of a reference sequence can vary widely from a full-length genome, to an individual chromosome, episome, gene, component of a gene, such as an exon, intron or

regulatory sequences, to a few nucleotides. A reference sequence of between about 2, 5, 10, 20, 50, 100, 500, 1000, 5,000 or 10,000, 20,000 or 100,000 nucleotides is common. Sometimes only particular regions of a sequence (e.g., exons
5 of a gene) are of interest. In such situations, the particular regions can be considered as separate reference sequences or can be considered as components of a single reference sequence, as matter of arbitrary choice.

A reference sequence can be any naturally occurring,
10 mutant, consensus or purely hypothetical sequence of nucleotides, RNA or DNA. For example, sequences can be obtained from computer data bases, publications or can be determined or conceived *de novo*. Usually, a reference sequence is selected to show a high degree of sequence
15 identity to envisaged target sequences. Often, particularly, where a significant degree of divergence is anticipated between target sequences, more than one reference sequence is selected. Combinations of wildtype and mutant reference sequences are employed in several applications of the tiling
20 strategy.

B. Chip Design

1. Basic Tiling Strategy

The basic tiling strategy provides an array of
25 immobilized probes for analysis of target sequences showing a high degree of sequence identity to one or more selected reference sequences. The strategy is first illustrated for an array that is subdivided into four probe sets, although it will be apparent that in some situations, satisfactory results
30 are obtained from only two probe sets. A first probe set comprises a plurality of probes exhibiting perfect complementarity with a selected reference sequence. The perfect complementarity usually exists throughout the length of the probe. However, probes having a segment or segments of
35 perfect complementarity that is/are flanked by leading or trailing sequences lacking complementarity to the reference sequence can also be used. Within a segment of complementarity, each probe in the first probe set has at

least one interrogation position that corresponds to a nucleotide in the reference sequence. That is, the interrogation position is aligned with the corresponding nucleotide in the reference sequence, when the probe and reference sequence are aligned to maximize complementarity between the two. If a probe has more than one interrogation position, each corresponds with a respective nucleotide in the reference sequence. The identity of an interrogation position and corresponding nucleotide in a particular probe in the first probe set cannot be determined simply by inspection of the probe in the first set. As will become apparent, an interrogation position and corresponding nucleotide is defined by the comparative structures of probes in the first probe set and corresponding probes from additional probe sets.

In principle, a probe could have an interrogation position at each position in the segment complementary to the reference sequence. Sometimes, interrogation positions provide more accurate data when located away from the ends of a segment of complementarity. Thus, typically a probe having a segment of complementarity of length x does not contain more than $x-2$ interrogation positions. Since probes are typically 9-21 nucleotides, and usually all of a probe is complementary, a probe typically has 1-19 interrogation positions. Often the probes contain a single interrogation position, at or near the center of probe.

For each probe in the first set, there are, for purposes of the present illustration, three corresponding probes from three additional probe sets. See Fig. 1. Thus, there are four probes corresponding to each nucleotide of interest in the reference sequence. Each of the four corresponding probes has an interrogation position aligned with that nucleotide of interest. Usually, the probes from the three additional probe sets are identical to the corresponding probe from the first probe set with one exception. The exception is that at least one (and often only one) interrogation position, which occurs in the same position in each of the four corresponding probes from the four probe sets, is occupied by a different nucleotide in the four probe sets. For example, for an A

nucleotide in the reference sequence, the corresponding probe from the first probe set has its interrogation position occupied by a T, and the corresponding probes from the additional three probe sets have their respective
5 interrogation positions occupied by A, C, or G, a different nucleotide in each probe. Of course, if a probe from the first probe set comprises trailing or flanking sequences lacking complementarity to the reference sequences (see Fig. 2), these sequences need not be present in corresponding
10 probes from the three additional sets. Likewise corresponding probes from the three additional sets can contain leading or trailing sequences outside the segment of complementarity that are not present in the corresponding probe from the first probe set. Occasionally, the probes from the additional three
15 probe set are identical (with the exception of interrogation position(s)) to a contiguous subsequence of the full complementary segment of the corresponding probe from the first probe set. In this case, the subsequence includes the interrogation position and usually differs from the full-
20 length probe only in the omission of one or both terminal nucleotides from the termini of a segment of complementarity. That is, if a probe from the first probe set has a segment of complementarity of length n , corresponding probes from the other sets will usually include a subsequence of the segment
25 of at least length $n-2$. Thus, the subsequence is usually at least 3, 4, 7, 9, 15, 21, or 25 nucleotides long, most typically, in the range of 9-21 nucleotides. The subsequence should be sufficiently long to allow a probe to hybridize detectably more strongly to a variant of the reference
30 sequence mutated at the interrogation position than to the reference sequence.

The probes can be oligodeoxyribonucleotides or oligoribonucleotides, or any modified forms of these polymers that are capable of hybridizing with a target nucleic sequence
35 by complementary base-pairing. Complementary base pairing means sequence-specific base pairing which includes e.g., Watson-Crick base pairing as well as other forms of base pairing such as Hoogsteen base pairing. Modified forms.

include 2'-O-methyl oligoribonucleotides and so-called PNAs, in which oligodeoxyribonucleotides are linked via peptide bonds rather than phosphodiester bonds. The probes can be attached by any linkage to a support (e.g., 3', 5' or via the
5 base). 3' attachment is more usual as this orientation is compatible with the preferred chemistry for solid phase synthesis of oligonucleotides.

The number of probes in the first probe set (and as a consequence the number of probes in additional probe sets)
10 depends on the length of the reference sequence, the number of nucleotides of interest in the reference sequence and the number of interrogation positions per probe. In general, each nucleotide of interest in the reference sequence requires the same interrogation position in the four sets of probes.
15 Consider, as an example, a reference sequence of 100 nucleotides, 50 of which are of interest, and probes each having a single interrogation position. In this situation, the first probe set requires fifty probes, each having one interrogation position corresponding to a nucleotide of
20 interest in the reference sequence. The second, third and fourth probe sets each have a corresponding probe for each probe in the first probe set, and so each also contains a total of fifty probes. The identity of each nucleotide of interest in the reference sequence is determined by comparing
25 the relative hybridization signals at four probes having interrogation positions corresponding to that nucleotide from the four probe sets.

In some reference sequences, every nucleotide is of interest. In other reference sequences, only certain portions
30 in which variants (e.g., mutations or polymorphisms) are concentrated are of interest. In other reference sequences, only particular mutations or polymorphisms and immediately adjacent nucleotides are of interest. Usually, the first probe set has interrogation positions selected to correspond
35 to at least a nucleotide (e.g., representing a point mutation) and one immediately adjacent nucleotide. Usually, the probes in the first set have interrogation positions corresponding to at least 3, 10, 50, 100, 1000, or 20,000 contiguous

nucleotides. The probes usually have interrogation positions corresponding to at least 5, 10, 30, 50, 75, 90, 99 or sometimes 100% of the nucleotides in a reference sequence. Frequently, the probes in the first probe set completely span the reference sequence and overlap with one another relative to the reference sequence. For example, in one common arrangement each probe in the first probe set differs from another probe in that set by the omission of a 3' base complementary to the reference sequence and the acquisition of a 5' base complementary to the reference sequence. See Fig. 3.

For conceptual simplicity, the probes in a set are usually arranged in order of the sequence in a lane across the chip. A lane contains a series of overlapping probes, which represent or tile across, the selected reference sequence (see Fig. 3). The components of the four sets of probes are usually laid down in four parallel lanes, collectively constituting a row in the horizontal direction and a series of 4-member columns in the vertical direction. Corresponding probes from the four probe sets (i.e., complementary to the same subsequence of the reference sequence) occupy a column. Each probe in a lane usually differs from its predecessor in the lane by the omission of a base at one end and the inclusion of additional base at the other end as shown in Fig. 3. However, this orderly progression of probes can be interrupted by the inclusion of control probes or omission of probes in certain columns of the array. Such columns serve as controls to orient the chip, or gauge the background, which can include target sequence nonspecifically bound to the chip.

The probes sets are usually laid down in lanes such that all probes having an interrogation position occupied by an A form an A-lane, all probes having an interrogation position occupied by a C form a C-lane, all probes having an interrogation position occupied by a G form a G-lane, and all probes having an interrogation position occupied by a T (or U) form a T lane (or a U lane). Note that in this arrangement there is not a unique correspondence between probe sets and lanes. Thus, the probe from the first probe set is laid down

in the A-lane, C-lane, A-lane, A-lane and T-lane for the five columns in Fig. 4. The interrogation position on a column of probes corresponds to the position in the target sequence whose identity is determined from analysis of hybridization to the probes in that column. Thus, I_1 - I_5 respectively correspond to N_1 - N_5 in Fig. 4. The interrogation position can be anywhere in a probe but is usually at or near the central position of the probe to maximize differential hybridization signals between a perfect match and a single-base mismatch. For example, for an 11 mer probe, the central position is the sixth nucleotide.

Although the array of probes is usually laid down in rows and columns as described above, such a physical arrangement of probes on the chip is not essential. Provided that the spatial location of each probe in an array is known, the data from the probes can be collected and processed to yield the sequence of a target irrespective of the physical arrangement of the probes on a chip. In processing the data, the hybridization signals from the respective probes can be reassorted into any conceptual array desired for subsequent data reduction whatever the physical arrangement of probes on the chip.

A range of lengths of probes can be employed in the chips. As noted above, a probe may consist exclusively of a complementary segments, or may have one or more complementary segments juxtaposed by flanking, trailing and/or intervening segments. In the latter situation, the total length of complementary segment(s) is more important than the length of the probe. In functional terms, the complementarity segment(s) of the first probe sets should be sufficiently long to allow the probe to hybridize detectably more strongly to a reference sequence compared with a variant of the reference including a single base mutation at the nucleotide corresponding to the interrogation position of the probe. Similarly, the complementarity segment(s) in corresponding probes from additional probe sets should be sufficiently long to allow a probe to hybridize detectably more strongly to a variant of the reference sequence having a single nucleotide

substitution at the interrogation position relative to the reference sequence. A probe usually has a single complementary segment having a length of at least 3 nucleotides, and more usually at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25 or 30 bases exhibiting perfect complementarity (other than possibly at the interrogation position(s) depending on the probe set) to the reference sequence. In bridging strategies, where more than one segment of complementarity is present, each segment provides at least three complementary nucleotides to the reference sequence and the combined segments provide at least two segments of three or a total of six complementary nucleotides. As in the other strategies, the combined length of complementary segments is typically from 6-30 nucleotides, and preferably from about 9-21 nucleotides. The two segments are often approximately the same length. Often, the probes (or segment of complementarity within probes) have an odd number of bases, so that an interrogation position can occur in the exact center of the probe.

In some chips, all probes are the same length. Other chips employ different groups of probe sets, in which case the probes are of the same size within a group, but differ between different groups. For example, some chips have one group comprising four sets of probes as described above in which all the probes are 11 mers, together with a second group comprising four sets of probes in which all of the probes are 13 mers. Of course, additional groups of probes can be added. Thus, some chips contain, e.g., four groups of probes having sizes of 11 mers, 13 mers, 15 mers and 17 mers. Other chips have different size probes within the same group of four probe sets. In these chips, the probes in the first set can vary in length independently of each other. Probes in the other sets are usually the same length as the probe occupying the same column from the first set. However, occasionally different lengths of probes can be included at the same column position in the four lanes. The different length probes are included to equalize hybridization signals from probes irrespective of

whether A-T or C-G bonds are formed at the interrogation position.

The length of probe can be important in distinguishing between a perfectly matched probe and probes showing a single-base mismatch with the target sequence. The discrimination is usually greater for short probes. Shorter probes are usually also less susceptible to formation of secondary structures. However, the absolute amount of target sequence bound, and hence the signal, is greater for larger probes. The probe length representing the optimum compromise between these competing considerations may vary depending on *inter alia* the GC content of a particular region of the target DNA sequence, secondary structure, synthesis efficiency and cross-hybridization. In some regions of the target, depending on hybridization conditions, short probes (e.g., 11 mers) may provide information that is inaccessible from longer probes (e.g., 19 mers) and vice versa. Maximum sequence information can be read by including several groups of different sized probes on the chip as noted above. However, for many regions of the target sequence, such a strategy provides redundant information in that the same sequence is read multiple times from the different groups of probes. Equivalent information can be obtained from a single group of different sized probes in which the sizes are selected to maximize readable sequence at particular regions of the target sequence. The appropriate size of probes at different regions of the target sequence can be determined from, e.g., Fig. 12, which compares the readability of different sized probes in different regions of a target. The strategy of customizing probe length within a single group of probe sets minimizes the total number of probes required to read a particular target sequence. This leaves ample capacity for the chip to include probes to other reference sequences.

The invention provides an optimization block which allows systematic variation of probe length and interrogation position to optimize the selection of probes for analyzing a particular nucleotide in a reference sequence. The block comprises alternating columns of probes complementary to the

wildtype target and probes complementary to a specific mutation. The interrogation position is varied between columns and probe length is varied down a column.

Hybridization of the chip to the reference sequence or the mutant form of the reference sequence identifies the probe length and interrogation position providing the greatest differential hybridization signal.

The probes are designed to be complementary to either strand of the reference sequence (e.g., coding or non-coding). Some chips contain separate groups of probes, one complementary to the coding strand, the other complementary to the noncoding strand. Independent analysis of coding and noncoding strands provides largely redundant information. However, the regions of ambiguity in reading the coding strand are not always the same as those in reading the noncoding strand. Thus, combination of the information from coding and noncoding strands increases the overall accuracy of sequencing.

Some chips contain additional probes or groups of probes designed to be complementary to a second reference sequence. The second reference sequence is often a subsequence of the first reference sequence bearing one or more commonly occurring mutations or interstrain variations. The second group of probes is designed by the same principles as described above except that the probes exhibit complementarity to the second reference sequence. The inclusion of a second group is particularly useful for analyzing short subsequences of the primary reference sequence in which multiple mutations are expected to occur within a short distance commensurate with the length of the probes (i.e., two or more mutations within 9 to 21 bases). Of course, the same principle can be extended to provide chips containing groups of probes for any number of reference sequences. Alternatively, the chips may contain additional probe(s) that do not form part of a tiled array as noted above, but rather serves as probe(s) for a conventional reverse dot blot. For example, the presence of mutation can be detected from binding of a target sequence to a single oligomeric probe harboring the mutation. Preferably, an

additional probe containing the equivalent region of the wildtype sequence is included as a control.

The chips are read by comparing the intensities of labelled target bound to the probes in an array.

5 Specifically, a comparison is performed between each lane of probes (e.g., A, C, G and T lanes) at each columnar position (physical or conceptual). For a particular columnar position, the lane showing the greatest hybridization signal is called as the nucleotide present at the position in the target
10 sequence corresponding to the interrogation position in the probes. See Fig. 5. The corresponding position in the target sequence is that aligned with the interrogation position in corresponding probes when the probes and target are aligned to maximize complementarity. Of the four probes in a column,
15 only one can exhibit a perfect match to the target sequence whereas the others usually exhibit at least a one base pair mismatch. The probe exhibiting a perfect match usually produces a substantially greater hybridization signal than the other three probes in the column and is thereby easily
20 identified. However, in some regions of the target sequence, the distinction between a perfect match and a one-base mismatch is less clear. Thus, a call ratio is established to define the ratio of signal from the best hybridizing probes to the second best hybridizing probe that must be exceeded for a
25 particular target position to be read from the probes. A high call ratio ensures that few if any errors are made in calling target nucleotides, but can result in some nucleotides being scored as ambiguous, which could in fact be accurately read. A lower call ratio results in fewer ambiguous calls, but can
30 result in more erroneous calls. It has been found that at a call ratio of 1.2 virtually all calls are accurate. However, a small but significant number of bases (e.g., up to about 10%) may have to be scored as ambiguous.

Although small regions of the target sequence can
35 sometimes be ambiguous, these regions usually occur at the same or similar segments in different target sequences. Thus, for precharacterized mutations, it is known in advance whether

that mutation is likely to occur within a region of unambiguously determinable sequence.

An array of probes is most useful for analyzing the reference sequence from which the probes were designed and variants of that sequence exhibiting substantial sequence similarity with the reference sequence (e.g., several single-base mutants spaced over the reference sequence). When an array is used to analyze the exact reference sequence from which it was designed, one probe exhibits a perfect match to the reference sequence, and the other three probes in the same column exhibits single-base mismatches. Thus, discrimination between hybridization signals is usually high and accurate sequence is obtained. High accuracy is also obtained when an array is used for analyzing a target sequence comprising a variant of the reference sequence that has a single mutation relative to the reference sequence, or several widely spaced mutations relative to the reference sequence. At different mutant loci, one probe exhibits a perfect match to the target, and the other three probes occupying the same column exhibit single-base mismatches, the difference (with respect to analysis of the reference sequence) being the lane in which the perfect match occurs.

For target sequences showing a high degree of divergence from the reference strain or incorporating several closely spaced mutations from the reference strain, a single group of probes (i.e., designed with respect to a single reference sequence) will not always provide accurate sequence for the highly variant region of this sequence. At some particular columnar positions, it may be that no single probe exhibits perfect complementarity to the target and that any comparison must be based on different degrees of mismatch between the four probes. Such a comparison does not always allow the target nucleotide corresponding to that columnar position to be called. Deletions in target sequences can be detected by loss of signal from probes having interrogation positions encompassed by the deletion. However, signal may also be lost from probes having interrogation positions closely proximal to the deletion resulting in some regions of the target sequence

that cannot be read. Target sequence bearing insertions will also exhibit short regions including and proximal to the insertion that usually cannot be read.

5 The presence of short regions of difficult-to-read target because of closely spaced mutations, insertions or deletion, does not prevent determination of the remaining sequence of the target as different regions of a target sequence are determined independently. Moreover, such ambiguities as might result from analysis of diverse variants with a single group
10 of probes can be avoided by including multiple groups of probe sets on a chip. For example, one group of probes can be designed based on a full-length reference sequence, and the other groups on subsequences of the reference sequence incorporating frequently occurring mutations or strain
15 variations.

A particular advantage of the present sequencing strategy over conventional sequencing methods is the capacity simultaneously to detect and quantify proportions of multiple target sequences. Such capacity is valuable, e.g., for
20 diagnosis of patients who are heterozygous with respect to a gene or who are infected with a virus, such as HIV, which is usually present in several polymorphic forms. Such capacity is also useful in analyzing targets from biopsies of tumor cells and surrounding tissues. The presence of multiple
25 target sequences is detected from the relative signals of the four probes at the array columns corresponding to the target nucleotides at which diversity occurs. The relative signals at the four probes for the mixture under test are compared with the corresponding signals from a homogeneous reference
30 sequence. An increase in a signal from a probe that is mismatched with respect to the reference sequence, and a corresponding decrease in the signal from the probe which is matched with the reference sequence signal the presence of a mutant strain in the mixture. The extent in shift in
35 hybridization signals of the probes is related to the proportion of a target sequence in the mixture. Shifts in relative hybridization signals can be quantitatively related to proportions of reference and mutant sequence by prior

calibration of the chip with seeded mixtures of the mutant and reference sequences. By this means, a chip can be used to detect variant or mutant strains constituting as little as 1, 5, 20, or 25 % of a mixture of stains.

5 Similar principles allow the simultaneous analysis of multiple target sequences even when none is identical to the reference sequence. For example, with a mixture of two target sequences bearing first and second mutations, there would be a variation in the hybridization patterns of probes having
10 interrogation positions corresponding to the first and second mutations relative to the hybridization pattern with the reference sequence. At each position, one of the probes having a mismatched interrogation position relative to the reference sequence would show an increase in hybridization
15 signal, and the probe having a matched interrogation position relative to the reference sequence would show a decrease in hybridization signal. Analysis of the hybridization pattern of the mixture of mutant target sequences, preferably in comparison with the hybridization pattern of the reference
20 sequence, indicates the presence of two mutant target sequences, the position and nature of the mutation in each strain, and the relative proportions of each strain.

 In a variation of the above method, the different components in a mixture of target sequences are differentially
25 labelled before being applied to the array. For example, a variety of fluorescent labels emitting at different wavelength are available. The use of differential labels allows independent analysis of different targets bound simultaneously to the array. For example, the methods permit comparison of
30 target sequences obtained from a patient at different stages of a disease.

2. Omission of Probes

 The general strategy outlined above employs four probes
35 to read each nucleotide of interest in a target sequence. One probe (from the first probe set) shows a perfect match to the reference sequence and the other three probes (from the second, third and fourth probe sets) exhibit a mismatch with

the reference sequence and a perfect match with a target sequence bearing a mutation at the nucleotide of interest. The provision of three probes from the second, third and fourth probe sets allows detection of each of the three possible nucleotide substitutions of any nucleotide of interest. However, in some reference sequences or regions of reference sequences, it is known in advance that only certain mutations are likely to occur. Thus, for example, at one site it might be known that an A nucleotide in the reference sequence may exist as a T mutant in some target sequences but is unlikely to exist as a C or G mutant. Accordingly, for analysis of this region of the reference sequence, one might include only the first and second probe sets, the first probe set exhibiting perfect complementarity to the reference sequence, and the second probe set having an interrogation position occupied by an invariant A residue (for detecting the T mutant). In other situations, one might include the first, second and third probes sets (but not the fourth) for detection of a wildtype nucleotide in the reference sequence and two mutant variants thereof in target sequences. In some chips, probes that would detect silent mutations (i.e., not affecting amino acid sequence) are omitted.

In some chips, the probes from the first probe set are omitted corresponding to some or all positions of the reference sequences. Such chips comprise at least two probe sets. The first probe set has a plurality of probes. Each probe comprises a segment exactly complementary to a subsequence of a reference sequence except in at least one interrogation position. A second probe set has a corresponding probe for each probe in the first probe set. The corresponding probe in the second probe set is identical to a sequence comprising the corresponding probe from the first probe set or a subsequence thereof that includes the at least one (and usually only one) interrogation position except that the at least one interrogation position is occupied by a different nucleotide in each of the two corresponding probes from the first and second probe sets. A third probe set, if present, also comprises a corresponding probe for each probe

in the first probe set except at the at least one interrogation position, which differs in the corresponding probes from the three sets. Omission of probes having a segment exhibiting perfect complementarity to the reference sequence results in loss of control information, i.e., the detection of nucleotides in a target sequence that are the same as those in a reference sequence. However, similar information can be obtained by hybridizing a chip lacking probes from the first probe set to both target and reference sequences. The hybridization can be performed sequentially, or concurrently, if the target and reference are differentially labelled. In this situation, the presence of a mutation is detected by a shift in the background hybridization intensity of the reference sequence to a perfectly matched hybridization signal of the target sequence, rather than by a comparison of the hybridization intensities of probes from the first set with corresponding probes from the second, third and fourth sets.

20 3. Wildtype Probe Lane

When the chips comprise four probe sets, as discussed *supra*, and the probe sets are laid down in four lanes, an A lane, a C-lane, a G lane and a T or U lane, the probe having a segment exhibiting perfect complementarity to a reference sequence varies between the four lanes from one column to another. This does not present any significant difficulty in computer analysis of the data from the chip. However, visual inspection of the hybridization pattern of the chip is sometimes facilitated by provision of an extra lane of probes, in which each probe has a segment exhibiting perfect complementarity to the reference sequence. See Fig. 4. This segment is identical to a segment from one of the probes in the other four lanes (which lane depending on the column position). The extra lane of probes (designated the wildtype lane) hybridizes to a target sequence at all nucleotide positions except those in which deviations from the reference sequence occurs. The hybridization pattern of the wildtype lane thereby provides a simple visual indication of mutations.

4. Deletion, Insertion and Multiple-Mutation Probes

Some chips provide an additional probe set specifically designed for analyzing deletion mutations. The additional probe set comprises a probe corresponding to each probe in the first probe set as described above. However, a probe from the additional probe set differs from the corresponding probe in the first probe set in that the nucleotide occupying the interrogation position is deleted in the probe from the additional probe set. See Fig. 6. Optionally, the probe from the additional probe set bears an additional nucleotide at one of its termini relative to the corresponding probe from the first probe set. The probe from the additional probe set will hybridize more strongly than the corresponding probe from the first probe set to a target sequence having a single base deletion at the nucleotide corresponding to the interrogation position. Additional probe sets are provided in which not only the interrogation position, but also an adjacent nucleotide is detected.

Similarly, other chips provide additional probe sets for analyzing insertions. For example, one additional probe set has a probe corresponding to each probe in the first probe set as described above. However, the probe in the additional probe set has an extra T nucleotide inserted adjacent to the interrogation position. See Fig. 6. Optionally, the probe has one fewer nucleotide at one of its termini relative to the corresponding probe from the first probe set. The probe from the additional probe set hybridizes more strongly than the corresponding probe from the first probe set to a target sequence having an A nucleotide inserted in a position adjacent to that corresponding to the interrogation position. Similar additional probe sets are constructed having C, G or T/U nucleotides inserted adjacent to the interrogation position. Usually, four such probe sets, one for each nucleotide, are used in combination.

Other chips provide additional probes (multiple-mutation probes) for analyzing target sequences having multiple closely spaced mutations. A multiple-mutation probe is usually identical to a corresponding probe from the first set as

described above, except in the base occupying the interrogation position, and except at one or more additional positions, corresponding to nucleotides in which substitution may occur in the reference sequence. The one or more additional positions in the multiple mutation probe are occupied by nucleotides complementary to the nucleotides occupying corresponding positions in the reference sequence when the possible substitutions have occurred.

5. Block Tiling

As noted in the discussion of the general tiling strategy, a probe in the first probe set sometimes has more than one interrogation position. In this situation, a probe in the first probe set is sometimes matched with multiple groups of at least one, and usually, three additional probe sets. See Fig. 7. Three additional probe sets are used to allow detection of the three possible nucleotide substitutions at any one position. If only certain types of substitution are likely to occur (e.g., transitions), only one or two additional probe sets are required (analogous to the use of probes in the basic tiling strategy). To illustrate for the situation where a group comprises three additional probe sets, a first such group comprises second, third and fourth probe sets, each of which has a probe corresponding to each probe in the first probe set. The corresponding probes from the second, third and fourth probes sets differ from the corresponding probe in the first set at a first of the interrogation positions. Thus, the relative hybridization signals from corresponding probes from the first, second, third and fourth probe sets indicate the identity of the nucleotide in a target sequence corresponding to the first interrogation position. A second group of three probe sets (designated fifth, sixth and seventh probe sets), each also have a probe corresponding to each probe in the first probe set. These corresponding probes differ from that in the first probe set at a second interrogation position. The relative hybridization signals from corresponding probes from the first, fifth, sixth, and seventh probe sets indicate the identity of the nucleotide in the target sequence

corresponding to the second interrogation position. As noted above, the probes in the first probe set often have seven or more interrogation positions. If there are seven
5 additional probe sets, each group of three probe sets serving to identify the nucleotide corresponding to one of the seven interrogation positions.

Each block of probes allows short regions of a target sequence to be read. For example, for a block of probes
10 having seven interrogation positions, seven nucleotides in the target sequence can be read. Of course, a chip can contain any number of blocks depending on how many nucleotides of the target are of interest. The hybridization signals for each block can be analyzed independently of any other block. The
15 block tiling strategy can also be combined with other tiling strategies, with different parts of the same reference sequence being tiled by different strategies.

The block tiling strategy offers two advantages over the basic strategy in which each probe in the first set has a
20 single interrogation position. One advantage is that the same sequence information can be obtained from fewer probes. A second advantage is that each of the probes constituting a block (i.e., a probe from the first probe set and a corresponding probe from each of the other probe sets) can
25 have identical 3' and 5' sequences, with the variation confined to a central segment containing the interrogation positions. The identity of 3' sequence between different probes simplifies the strategy for solid phase synthesis of the probes on the chip and results in more uniform deposition
30 of the different probes on the chip, thereby in turn increasing the uniformity of signal to noise ratio for different regions of the chip. A third advantage is that greater signal uniformity is achieved within a block.

35 6. Multiplex Tiling

In the block tiling strategy discussed above, the identity of a nucleotide in a target or reference sequence is determined by comparison of hybridization patterns of one

probe having a segment showing a perfect match with that of other probes (usually three other probes) showing a single base mismatch. In multiplex tiling, the identity of at least two nucleotides in a reference or target sequence is

5 determined by comparison of hybridization signal intensities of four probes, two of which have a segment showing perfect complementarity or a single base mismatch to the reference sequence, and two of which have a segment showing perfect complementarity or a double-base mismatch to a segment. The

10 four probes whose hybridization patterns are to be compared each have a segment that is exactly complementary to a reference sequence except at two interrogation positions, in which the segment may or may not be complementary to the reference sequence. The interrogation positions correspond to

15 the nucleotides in a reference or target sequence which are determined by the comparison of intensities. The nucleotides occupying the interrogation positions in the four probes are selected according to the following rule. The first interrogation position is occupied by a different nucleotide

20 in each of the four probes. The second interrogation position is also occupied by a different nucleotide in each of the four probes. In two of the four probes, designated the first and second probes, the segment is exactly complementary to the reference sequence except at not more than one of the two

25 interrogation positions. In other words, one of the interrogation positions is occupied by a nucleotide that is complementary to the corresponding nucleotide from the reference sequence and the other interrogation position may or may not be so occupied. In the other two of the four probes,

30 designated the third and fourth probes, the segment is exactly complementary to the reference sequence except that both interrogation positions are occupied by nucleotides which are noncomplementary to the respective corresponding nucleotides in the reference sequence.

35 There are number of ways of satisfying these conditions depending on whether the two nucleotides in the reference sequence corresponding to the two interrogation positions are the same or different. If these two nucleotides are different

in the reference sequence (probability 3/4), the conditions are satisfied by each of the two interrogation positions being occupied by the same nucleotide in any given probe. For example, in the first probe, the two interrogation positions
5 would both be A, in the second probe, both would be C, in the third probe, each would be G, and in the fourth probe each would be T or U. If the two nucleotides in the reference sequence corresponding to the two interrogation positions are different, the conditions noted above are satisfied by each of
10 the interrogation positions in any one of the four probes being occupied by complementary nucleotides. For example, in the first probe, the interrogation positions could be occupied by A and T, in the second probe by C and G, in the third probe by G and C, and in the fourth probe, by T and A. See (Fig. 8).

15 When the four probes are hybridized to a target that is the same as the reference sequence or differs from the reference sequence at one (but not both) of the interrogation positions, two of the four probes show a double-mismatch with the target and two probes show a single mismatch. The
20 identity of probes showing these different degrees of mismatch can be determined from the different hybridization signals. From the identity of the probes showing the different degrees of mismatch, the nucleotides occupying both of the interrogation positions in the target sequence can be deduced.

25 For ease of illustration, the multiplex strategy has been initially described for the situation where there are two nucleotides of interest in a reference sequence and only four probes in an array. Of course, the strategy can be extended to analyze any number of nucleotides in a target sequence by
30 using additional probes. In one variation, each pair of interrogation positions is read from a unique group of four probes. In a block variation, different groups of four probes exhibit the same segment of complementarity with the reference sequence, but the interrogation positions move within a block.
35 The block and standard multiplex tiling variants can of course be used in combination for different regions of a reference sequence. Either or both variants can also be used in combination with any of the other tiling strategies described.

7. Helper Mutations

Occasionally small regions of a reference sequence give a low hybridization signal as a result of annealing of probes. The self-annealing reduces the amount of probe effectively available for hybridizing to the target. Although such regions of the target are generally small and the reduction of hybridization signal is usually not so substantial as to obscure the sequence of this region, this concern can be avoided by the use of probes incorporating helper mutations. The helper mutation(s) serve to break-up regions of internal complementarity within a probe and thereby prevent annealing. Usually, one or two helper mutations are quite sufficient for this purpose. The inclusion of helper mutations can be beneficial in any of the tiling strategies noted above. In general each probe having a particular interrogation position has the same helper mutation(s). Thus, such probes have a segment in common which shows perfect complementarity with a reference sequence, except that the segment contains at least one helper mutation (the same in each of the probes) and at least one interrogation position (different in all of the probes). For example, in the basic tiling strategy, a probe from the first probe set comprises a segment containing an interrogation position and showing perfect complementarity with a reference sequence except for one or two helper mutations. The corresponding probes from the second, third and fourth probe sets usually comprise the same segment (or sometimes a subsequence thereof including the helper mutation(s) and interrogation position), except that the base occupying the interrogation position varies in each probe. See Fig. 9.

Usually, the helper mutation tiling strategy is used in conjunction with one of the tiling strategies described above. The probes containing helper mutations are used to tile regions of a reference sequence otherwise giving low hybridization signal (e.g., because of self-complementarity), and the alternative tiling strategy is used to tile intervening regions.

8. Pooling Strategies

Pooling strategies also employ arrays of immobilized probes. Probes are immobilized in cells of an array, and the hybridization signal of each cell can be determined independently of any other cell. A particular cell may be occupied by pooled mixture of probes. Although the identity of each probe in the mixture is known, the individual probes in the pool are not separately addressable. Thus, the hybridization signal from a cell is the aggregate of that of the different probes occupying the cell. In general, a cell is scored as hybridizing to a target sequence if at least one probe occupying the cell comprises a segment exhibiting perfect complementarity to the target sequence.

A simple strategy to show the increased power of pooled strategies over a standard tiling is to create three cells each containing a pooled probe having a single pooled position, the pooled position being the same in each of the pooled probes. At the pooled position, there are two possible nucleotide, allowing the pooled probe to hybridize to two target sequences. In tiling terminology, the pooled position of each probe is an interrogation position. As will become apparent, comparison of the hybridization intensities of the pooled probes from the three cells reveals the identity of the nucleotide in the target sequence corresponding to the interrogation position (i.e., that is matched with the interrogation position when the target sequence and pooled probes are maximally aligned for complementarity).

The three cells are assigned probe pools that are perfectly complementary to the target except at the pooled position, which is occupied by a different pooled nucleotide in each probe as follows:

[AC] = M, [GT]=K, [AG]=R
 as substitutions in the probe
 IUPAC standard ambiguity notation)

X - interrogation position

5 Target: TAACCACTCACGGGAGCA

Pool 1: ATTGGMGAGTGCCC
 =ATTGGAAGAGTGCCC (complement to mutant 't')
 +ATTGGCGAGTGCCC (complement to mutant 'g')

10 Pool 2: ATTGGKGAGTGCCC
 =ATTGGGgAGTGCCC (complement to mutant 'c')
 +ATTGGtGAGTGCCC (complement to wild type 'a')

15 Pool 3: ATTGGRGAGTGCCC
 =ATTGGAAGAGTGCCC (complement to mutant 't')
 +ATTGGgGAGTGCCC (complement to mutant 'c')

20 With 3 pooled probes, all 4 possible single base pair states
 (wild and 3 mutants) are detected. A pool hybridizes with a
 target if some probe contained within that pool is
 complementary to that target.

25	Pool:	Hybridization?		
		1	2	3
	Target: TAACCACTCACGGGAGCA	n	y	n
	Mutant: TAACCCCTCACGGGAGCA	n	y	y
	Mutant: TAACCGCTCACGGGAGCA	y	n	n
30	Mutant: TAACCTCTCACGGGAGCA	y	n	y

A cell containing a pair (or more) of oligonucleotides
 lights up when a target complementary to any of the
 oligonucleotide in the cell is present. Using the simple
 35 strategy, each of the four possible targets (wild and three
 mutants) yields a unique hybridization pattern among the three
 cells.

Since a different pattern of hybridizing pools is
 obtained for each possible nucleotide in the target sequence
 40 corresponding to the pooled interrogation position in the
 probes, the identity of the nucleotide can be determined from
 the hybridization pattern of the pools. Whereas, a standard
 tiling requires four cells to detect and identify the possible
 single-base substitutions at one location, this simple pooled
 45 strategy only requires three cells.

A more efficient pooling strategy for sequence analysis is the 'Trellis' strategy. In this strategy, each pooled probe has a segment of perfect complementarity to a reference sequence except at three pooled positions. One pooled position is an N pool. The three pooled positions may or may not be contiguous in a probe. The other two pooled positions are selected from the group of three pools consisting of (1) M or K, (2) R or Y and (3) W or S, where the single letters are IUPAC standard ambiguity codes. The sequence of a pooled probe is thus, of the form XXXN[(M/K) or (R/Y) or (W/S)][(M/K) or (R/Y) or (W/S)]XXXXX, where XXX represents bases complementary to the reference sequence. The three pooled positions may be in any order, and may be contiguous or separated by intervening nucleotides. For, the two positions occupied by [(M/K) or (R/Y) or (W/S)], two choices must be made. First, one must select one of the following three pairs of pooled nucleotides (1) M/K, (2) R/Y and (3) W/S. The one of three pooled nucleotides selected may be the same or different at the two pooled positions. Second, supposing, for example, one selects M/K at one position, one must then choose between M or K. This choice should result in selection of a pooled nucleotide comprising a nucleotide that complements the corresponding nucleotide in a reference sequence, when the probe and reference sequence are maximally aligned. The same principle governs the selection between R and Y, and between W and S. A trellis pool probe has one pooled position with four possibilities, and two pooled positions, each with two possibilities. Thus, a trellis pool probe comprises a mixture of 16 ($4 \times 2 \times 2$) probes. Since each pooled position includes one nucleotide that complements the corresponding nucleotide from the reference sequence, one of these 16 probes has a segment that is the exact complement of the reference sequence. A target sequence that is the same as the reference sequence (i.e., a wildtype target) gives a hybridization signal to each probe cell. Here, as in other tiling methods, the segment of complementarity should be sufficiently long to permit specific hybridization of a pooled probe to a reference sequence be detected relative to a variant of that reference

sequence. Typically, the segment of complementarity is about 9-21 nucleotides.

A target sequence is analyzed by comparing hybridization intensities at three pooled probes, each having the structure described above. The segments complementary to the reference sequence present in the three pooled probes show some overlap. Sometimes the segments are identical (other than at the interrogation positions). However, this need not be the case. For example, the segments can tile across a reference sequence in increments of one nucleotide (i.e., one pooled probe differs from the next by the acquisition of one nucleotide at the 5' end and loss of a nucleotide at the 3' end). The three interrogation positions may or may not occur at the same relative positions within each pooled probe (i.e., spacing from a probe terminus). All that is required is that one of the three interrogation positions from each of the three pooled probes aligns with the same nucleotide in the reference sequence, and that this interrogation position is occupied by a different pooled nucleotide in each of the three probes. In one of the three probes, the interrogation position is occupied by an N. In the other two pooled probes the interrogation position is occupied by one of (M/K) or (R/Y) or (W/S).

In the simplest form of the trellis strategy, three pooled probes are used to analyze a single nucleotide in the reference sequence. Much greater economy of probes is achieved when more pooled probes are included in an array. For example, consider an array of five pooled probes each having the general structure outlined above. Three of these pooled probes have an interrogation position that aligns with the same nucleotide in the reference sequence and are used to read that nucleotide. A different combination of three probes have an interrogation position that aligns with a different nucleotide in the reference sequence. Comparison of these three probe intensities allows analysis of this second nucleotide. Still another combination of three pooled probes from the set of five have an interrogation position that aligns with a third nucleotide in the reference sequence and

these probes are used to analyze that nucleotide. Thus, three nucleotides in the reference sequence are fully analyzed from only five pooled probes. By comparison, the basic tiling strategy would require 12 probes for a similar analysis.

5 As an example, a pooled probe for analysis of a target sequence by the trellis strategy is shown below:

Target: ATTAACCACTCACGGGAGCTCT

Pool: TGGTGKNGGCCCT

10

The pooled probe actually comprises 16 individual probes:

 TGGTGAGcGCCCT
 +TGGTGcGcGCCCT
15 +TGGTGgGcGCCCT
 +TGGTGtGcGCCCT
 +TGGTGAtcGCCCT
 +TGGTGctcGCCCT
 +TGGTGgtcGCCCT
20 +TGGTGttcGCCCT
 +TGGTGAGTGCCCT
 +TGGTGcGTGCCCT
 +TGGTGgGTGCCCT
 +TGGTGtGTGCCCT
25 +TGGTGAtTGCCCT
 +TGGTGctTGCCCT
 +TGGTGgtTGCCCT
 +TGGTGttTGCCCT

30

The trellis strategy employs an array of probes having at least three cells, each of which is occupied by a pooled probe as described above.

35 Consider the use of three such pooled probes for analyzing a target sequence, of which one position may contain any single base substitution to the reference sequence (i.e., there are four possible target sequences to be distinguished). Three cells are occupied by pooled probes having a pooled interrogation position corresponding to the position of
40 possible substitution in the target sequence, one cell with an 'N', one cell with one of 'M' or 'K', and one cell with 'R' or 'Y'. An interrogation position corresponds to a nucleotide in the target sequence if it aligns adjacent with that nucleotide when the probe and target sequence are aligned to maximize
45 complementarity. Note that although each of the pooled

probes has two other pooled positions, these positions are not relevant for the present illustration. The positions are only relevant when more than one position in the target sequence is to be read, a circumstance that will be considered later. For present purposes, the cell with the 'N' in the interrogation position lights up for the wildtype sequence and any of the three single base substitutions of the target sequence. The cell with M/K in the interrogation position lights up for the wildtype sequence and one of the single-base substitutions. The cell with R/Y in the interrogation position lights up for the wildtype sequence and a second of the single-base substitutions. Thus, the four possible target sequences hybridize to the three pools of probes in four distinct patterns, and the four possible target sequences can be distinguished.

To illustrate further, consider four possible target sequences (differing at a single position) and a pooled probe having three pooled positions, N, K and Y with the Y position as the interrogation position (i.e., aligned with the variable position in the target sequence):

Target

Wild: ATTAACCACTCACGGGAGCTCT (w)
 Mutants: ATTAACCACTCccGGGAGCTCT (c)
 Mutants: ATTAACCACTCgCGGGAGCTCT (g)
 5 Mutants: ATTAACCACTctCGGGAGCTCT (t)
 TGGTGNKYGCCCT (pooled probe).

The sixteen individual component probes of the pooled probe hybridize to the four possible target sequences as follows:

		TARGET			
		w	c	g	t
10	TGGTGAGcGCCCT	n	n	y	n
	TGGTGcGcGCCCT	n	n	n	n
	TGGTGgGcGCCCT	n	n	n	n
15	TGGTGtGcGCCCT	n	n	n	n
	TGGTGatcGCCCT	n	n	n	n
	TGGTGctcGCCCT	n	n	n	n
	TGGTGgtcGCCCT	n	n	n	n
	TGGTGttcGCCCT	n	n	n	n
20	TGGTGAGTGCCCT	y	n	n	n
	TGGTGcGTGCCCT	n	n	n	n
	TGGTGgGTGCCCT	n	n	n	n
	TGGTGtGTGCCCT	n	n	n	n
	TGGTGatTGCCCT	n	n	n	n
25	TGGTGctTGCCCT	n	n	n	n
	TGGTGgtTGCCCT	n	n	n	n
	TGGTGttTGCCCT	n	n	n	n

The pooled probe hybridizes according to the aggregate of its components:

Pool: TGGTGNKYGCCCT y n y n

Thus, as stated above, it can be seen that a pooled probe having a y at the interrogation position hybridizes to the wildtype target and one of the mutants. Similar tables can be drawn to illustrate the hybridization patterns of probe pools having other pooled nucleotides at the interrogation position.

The above strategy of using pooled probes to analyze a single base in a target sequence can readily be extended to analyze any number of bases. At this point, the purpose of including three pooled positions within each probe will become apparent. In the example that follows, ten pools of probes, each containing three pooled probe positions, can be used to analyze a each of a contiguous sequence of eight nucleotides in a target sequence.

ATTAACCACTCACGGGAGCTCT Reference sequence
 ----- Readable nucleotides

Pools:
 5 4 TAATTNKYGAGTG
 5 AATTGNKRAGTGC
 6 ATTGGNKRGTGCC
 7 TTGGTNMRTGCCC
 8 TGGTGNKYGCCCT
 10 9 GGTGANKRCCCTC
 10 10 GTGAGNKYCCTCG
 11 TGAGTNMYCTCGA
 12 GAGTGNMYTCGAG
 13 AGTGCNMYCGAGA
 15

In this example, the different pooled probes tile across the reference sequence, each pooled probe differing from the next by increments of one nucleotide. For each of the readable nucleotides in the reference sequence, there are three probe pools having a pooled interrogation position aligned with the readable nucleotide. For example, the 12th nucleotide from the left in the reference sequence is aligned with pooled interrogation positions in pooled probes 8, 9, and 10. Comparison of the hybridization intensities of these pooled probes reveals the identity of the nucleotide occupying position 12 in a target sequence.

	Targets	Pools		
		8	9	10
30 Wild:	ATTAACCACTCACGGGAGCTCT	Y	Y	Y
Mutants:	ATTAACCACTCcCGGGAGCTCT	N	Y	Y
Mutants:	ATTAACCACTCgCGGGAGCTCT	Y	N	Y
35 Mutants:	ATTAACCACTCtCGGGAGCTCT	N	N	Y

Example Intensities:

	= lit cell	Wild				
	= blank cell	'C'				
40		'G'				
		'T'				
		None				

45 Thus, for example, if pools 8, 9 and 10 all light up, one knows the target sequence is wildtype, If pools, 9 and 10

light up, the target sequence has a C mutant at position 12. If pools 8 and 10 light up, the target sequence has a G mutant at position 12. If only pool 10 lights up, the target sequence has a t mutant at position 12.

5 The identity of other nucleotides in the target sequence is determined by a comparison of other sets of three pooled probes. For example, the identity of the 13th nucleotide in the target sequence is determined by comparing the hybridization patterns of the probe pools designated 9, 10 and 10 11. Similarly, the identity of the 14th nucleotide in the target sequence is determined by comparing the hybridization patterns of the probe pools designated 10, 11, and 12.

15 In the above example, successive probes tile across the reference sequence in increments of one nucleotide, and each probe has three interrogation positions occupying the same positions in each probe relative to the terminus of the probe (i.e., the 7, 8 and 9th positions relative to the 3' terminus). However, the trellis strategy does not require that probes tile in increments of one or that the 20 interrogation position positions occur in the same position in each probe. In a variant of trellis tiling referred to as "loop" tiling, a nucleotide of interest in a target sequence is read by comparison of pooled probes, which each have a pooled interrogation position corresponding to the nucleotide 25 of interest, but in which the spacing of the interrogation position in the probe differs from probe to probe. Analogously to the block tiling approach, this allows several nucleotides to be read from a target sequence from a collection of probes that are identical except at the 30 interrogation position. The identity in sequence of probes, particularly at their 3' termini, simplifies synthesis of the array and result in more uniform probe density per cell.

35 To illustrate the loop strategy, consider a reference sequence of which the 4, 5, 6, 7 and 8th nucleotides (from the 3' termini are to be read. All of the four possible nucleotides at each of these positions can be read from comparison of hybridization intensities of five pooled probes. Note that the pooled positions in the probes are different

(for example in probe 55, the pooled positions are 4, 5 and 6 and in probe 56, 5, 6 and 7).

		TAACCACTCACGGGAGCA	Reference sequence
55		ATTNKYGAGTGCC	
56		ATTGNKRAGTGCC	
57		ATTGGNKRGTGCC	
58		ATTRGTNMGTGCC	
59		ATTKRTGNGTGCC	

Each position of interest in the reference sequence is read by comparing hybridization intensities for the three probe pools that have an interrogation position aligned with the nucleotide of interest in the reference sequence. For example, to read the fourth nucleotide in the reference sequence, probes 55, 58 and 59 provide pools at the fourth position. Similarly, to read the fifth nucleotide in the reference sequence, probes 55, 56 and 59 provide pools at the fifth position. As in the previous trellis strategy, one of the three probes being compared has an N at the pooled position and the other two have M or K, and (2) R or Y and (3) W or S.

The hybridization pattern of the five pooled probes to target sequences representing each possible nucleotide substitution at five positions in the reference sequence is shown below. Each possible substitution results in a unique hybridization pattern at three pooled probes, and the identity of the nucleotide at that position can be deduced from the hybridization pattern.

		Targets	55	56	Pools 57	58	59
5	Wild:	TAACCACTCACGGGAGCA	Y	Y	Y	Y	Y
	Mutant:	TAAgCACTCACGGGAGCA	Y	N	N	N	N
	Mutant:	TAAtCACTCACGGGAGCA	Y	N	N	Y	N
	Mutant:	TAAaCACTCACGGGAGCA	Y	N	N	N	Y
10	Mutant:	TAACgACTCACGGGAGCA	N	Y	N	N	N
	Mutant:	TAActACTCACGGGAGCA	N	Y	N	N	Y
	Mutant:	TAAcAaCTCACGGGAGCA	Y	Y	N	N	N
15	Mutant:	TAACCCCTCACGGGAGCA	N	Y	Y	N	N
	Mutant:	TAACCGCTCACGGGAGCA	Y	N	Y	N	N
	Mutant:	TAACCTCTCACGGGAGCA	N	N	Y	N	N
	Mutant:	TAACCAgTCACGGGAGCA	N	N	N	Y	N
20	Mutant:	TAACCAtTCACGGGAGCA	N	Y	N	Y	N
	Mutant:	TAACCAaTCACGGGAGCA	N	N	Y	Y	N
	Mutant:	TAACCACaCACGGGAGCA	N	N	N	N	Y
	Mutant:	TAACCACcCACGGGAGCA	N	N	Y	N	Y
25	Mutant:	TAACCACgCACGGGAGCA	N	N	N	Y	Y

Many variations on the loop and trellis tilings can be created. All that is required is that each position in sequence must have a probe with a 'N', a probe containing one of R/Y, M/K or W/S, and a probe containing a different pool from that set, complementary to the wild type target at that position, and at least one probe with no pool at all at that position. This combination allows all mutations at that position to be uniquely detected and identified.

A further class of strategies involving pooled probes are termed coding strategies. These strategies assign code words from some set of numbers to variants of a reference sequence. Any number of variants can be coded. The variants can include multiple closely spaced substitutions, deletions or insertions. The designation letters or other symbols assigned to each variant may be any arbitrary set of numbers, in any order. For example, a binary code is often used, but codes to other bases are entirely feasible. The numbers are often assigned such that each variant has a designation having at least one digit and at least one nonzero value for that digit. For example, in a binary system, a variant assigned the number

101, has a designation of three digits, with one possible nonzero value for each digit.

The designation of the variants are coded into an array of pooled probes comprising a pooled probe for each nonzero value of each digit in the numbers assigned to the variants. For example, if the variants are assigned successive number in a numbering system of base m , and the highest number assigned to a variant has n digits, the array would have about $n \times (m-1)$ pooled probes. In general, $\log_m (3N+1)$ probes are required to analyze all variants of N locations in a reference sequence, each having three possible mutant substitutions. For example, 10 base pairs of sequence may be analyzed with only 5 pooled probes using a binary coding system. Each pooled probe has a segment exactly complementary to the reference sequence except that certain positions are pooled. The segment should be sufficiently long to allow specific hybridization of the pooled probe to the reference sequence relative to a mutated form of the reference sequence. As in other tiling strategies, segments lengths of 9-21 nucleotides are typical. Often the probe has no nucleotides other than the 9-21 nucleotide segment. The pooled positions comprise nucleotides that allow the pooled probe to hybridize to every variant assigned a particular nonzero value in a particular digit. Usually, the pooled positions further comprises a nucleotide that allows the pooled probe to hybridize to the reference sequence. Thus, a wildtype target (or reference sequence) is immediately recognizable from all the pooled probes being lit.

When a target is hybridized to the pools, only those pools comprising a component probe having a segment that is exactly complementary to the target light up. The identity of the target is then decoded from the pattern of hybridizing pools. Each pool that lights up is correlated with a particular value in a particular digit. Thus, the aggregate hybridization patterns of each lighting pool reveal the value of each digit in the code defining the identity of the target hybridized to the array.

As an example, consider a reference sequence having four positions, each of which can be occupied by three possible mutations. Thus, in total there are 4×3 possible variant forms of the reference sequence. Each variant is assigned a binary number binary numbers 0001-1100 and the wildtype reference sequence is assigned the binary number 1111.

		X	X	X	X	-	4
10	Positions						
	Target: TAAC	C=1111	A=1111	C=1111	T=1111		
	CACGGGAGCA						
		G=0001	C=0010	G=0011	A=0100		
		T=0101	G=0110	T=0111	C=1000		
		A=1001	T=1010	A=1011	G=1100		
15							

A first pooled probe is designed by including probes that complement exactly each variant having a 1 in the first digit.

20	target(1111):	TAAC	C	A	C	T	CACGGGAGCA
	Mutant(0001):	TAAC	g	A	C	T	CACGGGAGCA
	Mutant(0101):	TAAC	t	A	C	T	CACGGGAGCA
	Mutant(1001):	TAAC	a	A	C	T	CACGGGAGCA
25	Mutant(0011):	TAAC	C	A	g	T	CACGGGAGCA
	Mutant(0111):	TAAC	C	A	t	T	CACGGGAGCA
	Mutant(1101):	TAAC	C	A	a	T	CACGGGAGCA
	First pooled probe						
30	=	ATTG	[GCAT]	T	[GCAT]	A	GTGCCC
	=	ATTG	N	T	N	A	GTGCCC

Second, third and fourth pooled probes are then designed respectively including component probes that hybridize to each variant having a 1 in the second, third and fourth digit.

XXXX - 4 positions examined

40	Target:	TAACCACTCACGGGAGCA		
	Pool 1(1):	ATTGnTnAGTGCCC =	16 probes	(4x1x4x1)
	Pool 2(2):	ATTGGnnAGTGCCC =	16 probes	(1x4x4x1)
	Pool 3(4):	ATTGyrydGTGCCC =	24 probes	(2x2x2x3)
	Pool 4(8):	ATTGmwmbGTGCCC =	24 probes	(2x2x2x3)

The pooled probes hybridize to variant targets as follows:

Hybridization pattern:

	Targets	Pools			
		1	2	3	4
5	Wild(1111)	Y	Y	Y	Y
	Mutant(0001):	Y	N	N	N
	Mutant(0101):	Y	N	Y	N
	Mutant(1001):	Y	N	N	Y
10	Mutant(0010):	N	Y	N	N
	Mutant(0110):	N	Y	Y	N
	Mutant(1010):	N	Y	N	Y
15	Mutant(0011):	Y	Y	N	N
	Mutant(0111):	Y	Y	Y	N
	Mutant(1101):	Y	N	Y	Y
	Mutant(0100):	N	N	Y	N
20	Mutant(1000):	N	N	N	Y
	Mutant(1100):	N	N	Y	Y

The identity of a variant (i.e., mutant) target is read directly from the hybridization pattern of the pooled probes. For example the mutant assigned the number 0001 gives a hybridization pattern of NNNY with respect to probes 4, 3, 2 and 1 respectively.

In the above example, variants are assigned successive numbers in a numbering system. In other embodiments, sets of numbers can be chosen for their properties. If the codewords are chosen from an error-control code, the properties of that code carry over to sequence analysis. An error code is a numbering system in which some designations are assigned to variants and other designations serve to indicate errors that may have occurred in the hybridization process. For example, if all codewords have an odd number of nonzero digits ('binary coding+error detection'), any single error in hybridization will be detected by having an even number of pools lit.

40

Wild
Target: TAACCACTCACGGGAGCA

45	Pool 1(1):	ATTGnAnAGTGCCC =	16 Probes	(4x1x4x1)
	Pool 2(2):	ATTGGnnAGTGCCC =	16 Probes	(1X4X4X1)
	Pool 3(4):	ATTGryrhGTGCCC =	24 Probes	(2X2X2X3)
	Pool 4(8):	ATTGkwkvGTGCCC =	24 Probes	(2X2X2X3)

A fifth probe can be added to make the number of pools that hybridize to any single mutation odd.

Pool 5(c): ATTGdhsmGTGCCC = 36 probes (2x2x3x3)

5

Hybridization of pooled probes to targets

		Pool				
Target		1	2	3	4	5
10	Target(11111): TAACCACTCACGGGAGCA	Y	Y	Y	Y	Y
	Mutant(00001): TAACgACTCACGGGAGCA	Y	N	N	N	N
	Mutant(10101): TAActACTCACGGGAGCA	Y	N	N	N	N
	Mutant(11001): TAACaACTCACGGGAGCA	Y	N	N	Y	Y
15	Mutant(00010): TAACCCCTCACGGGAGCA	N	Y	N	N	N
	Mutant(10110): TAACCGCTCACGGGAGCA	N	Y	Y	N	Y
	Mutant(11010): TAACctCTCACGGGAGCA	N	Y	N	Y	Y
	Mutant(10011): TAACCAgTCACGGGAGCA	Y	Y	N	N	Y
20	Mutant(00111): TAACAtTCACGGGAGCA	Y	Y	Y	N	N
	Mutant(01101): TAACCAaTCACGGGAGCA	Y	N	Y	Y	N
	Mutant(00100): TAACCACaCACGGGAGCA	N	N	Y	N	N
	Mutant(01000): TAACCACCCACGGGAGCA	N	N	N	Y	N
25	Mutant(11100): TAACCACgCACGGGAGCA	N	N	Y	Y	Y

9. Bridging Strategy

Probes that contain partial matches to two separate
 30 (i.e., non contiguous) subsequences of a target sequence
 sometimes hybridize strongly to the target sequence. In
 certain instances, such probes have generated stronger signals
 than probes of the same length which are perfect matches to
 the target sequence. It is believed (but not necessary to the
 35 invention) that this observation results from interactions of
 a single target sequence with two or more probes
 simultaneously. This invention exploits this observation to
 provide arrays of probes having at least first and second
 segments, which are respectively complementary to first and
 40 second subsequences of a reference sequence. Optionally, the
 probes may have a third or more complementary segments. These
 probes can be employed in any of the strategies noted above.
 The two segments of such a probe can be complementary to
 disjoint subsequences of the reference sequences or contiguous
 45 subsequences. If the latter, the two segments in the probe
 are inverted relative to the order of the complement of the

reference sequence. The two subsequences of the reference sequence each typically comprises about 3 to 30 contiguous nucleotides. The subsequences of the reference sequence are sometimes separated by 0, 1, 2 or 3 bases. Often the sequences, are adjacent and nonoverlapping.

For example, a wild-type probe is created by complementing two sections of a reference sequence (indicated by subscript and superscript) and reversing their order. The interrogation position is designated (*) and is apparent from comparison of the structure of the wildtype probe with the three mutant probes. The corresponding nucleotide in the reference sequence is the "a" in the superscripted segment.

Reference: 5' T_{GGCTA}^{CGAGG}AATCATCTGTTA
 15
 *
 Probes: 3' GCTCC CCGAT (Probe from first probe set)
 3' GCACC CCGAT
 3' GCCCC CCGAT
 20 3' GCGCC CCGAT

The expected hybridizations are:

Match:
 25 GCTCCCCGAT
 ... TGGCTACGAGGAATCATCTGTTA
GCTCCCCGAT

Mismatch:
 30 GCTCCCCGAT
 ... TGGCTACGAGGAATCATCTGTTA
GCGCCCCGAT

Bridge tilings are specified using a notation which gives the length of the two constituent segments and the relative position of the interrogation position. The designation n/m indicates a segment complementary to a region of the reference sequence which extends for n bases and is located such that the interrogation position is in the mth base from the 5' end. If m is larger than n, this indicates that the entire segment is to the 5' side of the interrogation position. If m is negative, it indicates that the interrogation position is the absolute value of m bases 5' of the first base of the segment (m cannot be zero). Probes comprising multiple segments, such as n/m + a/b + ... have a first segment at the 3' end of the

probe and additional segments added 5' with respect to the first segment. For example, a 4/8 tiling consists of (from the 3' end of the probe) a 4 base complementary segment, starting 7 bases 5' of the interrogation position, followed by
 5 a 6 base region in which the interrogation position is located at the third base. Between these two segments, one base from the reference sequence is omitted. By this notation, the set shown above is a 5/3 + 5/8 tiling. Many different tilings are possible with this method, since the lengths of both segments
 10 can be varied, as well as their relative position (they may be in either order and there may be a gap between them) and their location relative to the interrogation position.

As an example, a 16 mer oligo target was hybridized to a chip containing all 4¹⁰ probes of length 10. The chip
 15 includes short tilings of both standard and bridging types. The data from a standard 10/5 tiling was compared to data from a 5/3 + 5/8 bridge tiling (see Table 1). Probe intensities (mean count/pixel) are displayed along with discrimination ratios (correct probe intensity / highest incorrect probe
 20 intensity). Missing intensity values are less than 50 counts. Note that for each base displayed the bridge tiling has a higher discrimination value.

25 TABLE 1: Comparison of Standard and Bridge Tilings

TILING	PROBE BASE:	CORRECT PROBE BASE			
		C	A	C	C
30 STANDARD (10/5)	A	92	496	294	299
	C	536	148	532	534
	G	69	167	72	52
	T	146	95	212	126
35 DISCRIMINATION:		3.7	3.0	1.8	1.8
40 BRIDGING 5/3 + 5/8	A	-	404	-	156
	C	276	-	345	379
	G	-	80	-	-
	T	-	-	-	58
45 DISCRIMINATION:		>5.5	5.1	2.4	1.26

The bridging strategy offers the following advantages:

(1) Higher discrimination between matched and mismatched probes,

(2) The possibility of using longer probes in a bridging tiling, thereby increasing the specificity of the hybridization, without sacrificing discrimination,

(3) The use of probes in which an interrogation position is located very off-center relative to the regions of target complementarity. This may be of particular advantage when, for example, when a probe centered about one region of the target gives low hybridization signal. The low signal is overcome by using a probe centered about an adjoining region giving a higher hybridization signal.

(4) Disruption of secondary structure that might result in annealing of certain probes (see previous discussion of helper mutations).

10. Deletion Tiling

Deletion tiling is related to both the bridging and helper mutant strategies described above. In the deletion strategy, comparisons are performed between probes sharing a common deletion but differing from each other at an interrogation position located outside the deletion. For example, a first probe comprises first and second segments, each exactly complementary to respective first and second subsequences of a reference sequence, wherein the first and second subsequences of the reference sequence are separated by a short distance (e.g., 1 or 2 nucleotides). The order of the first and second segments in the probe is usually the same as that of the complement to the first and second subsequences in the reference sequence. The interrogation position is usually separated from the comparison is performed with three other probes, which are identical to the first probe except at an interrogation position, which is different in each probe.

Reference: . . . AGTACCAGATCTCTAA . . .

Probe set: CATGGNC AGAGA (N = interrogation position).

Such tilings sometimes offer superior discrimination in hybridization intensities between the probe having an interrogation position complementary to the target and other probes. Thermodynamically, the difference between the hybridizations to matched and mismatched targets for the probe

set shown above is the difference between a single-base bulge, and a large asymmetric loop (e.g., two bases of target, one of probe). This often results in a larger difference in stability than the comparison of a perfectly matched probe with a probe showing a single base mismatch in the basic tiling strategy.

The superior discrimination offered by deletion tiling is illustrated by Table 2, which compares hybridization data from a standard 10/5 tiling with a (4/8 + 6/3) deletion tiling of the reference sequence. (The numerators indicate the length of the segments and the denominators, the spacing of the deletion from the far termini of the segments.) Probe intensities (mean count/pixel) are displayed along with discrimination ratios (correct probe intensity / highest incorrect probe intensity). Note that for each base displayed the deletion tiling has a higher discrimination value than either standard tiling shown.

TABLE 2. Comparison of Standard and Deletion Tilings

TILING	PROBE BASE:	CORRECT PROBE BASE			
		C	A	C	C
STANDARD (10/5)	A	92	496	294	299
	C	536	148	532	534
	G	69	167	72	52
	T	146	95	212	126
DISCRIMINATION:		3.7	3.0	1.8	1.8
DELETION 4/8 + 6/3	A	6	412	29	48
	C	297	32	465	160
	G	8	77	10	4
	T	8	26	31	5
DISCRIMINATION:		37.1	5.4	15	3.3
STANDARD (10/7)	A	347	533	228	277
	C	729	194	536	496
	G	232	231	102	89
	T	344	133	163	150
DISCRIMINATION:		2.1	2.3	2.3	1.8

The use of deletion or bridging probes is quite general. These probes can be used in any of the tiling strategies of the invention. As well as offering superior discrimination, the use of deletion or bridging strategies is advantageous for

certain probes to avoid self-hybridization (either within a probe or between two probes of the same sequence)

C. Preparation of Target Samples

5 The target polynucleotide, whose sequence is to be
determined, is usually isolated from a tissue sample. If the
target is genomic, the sample may be from any tissue (except
exclusively red blood cells). For example, whole blood,
peripheral blood lymphocytes or PBMC, skin, hair or semen are
10 convenient sources of clinical samples. These sources are
also suitable if the target is RNA. Blood and other body
fluids are also a convenient source for isolating viral
nucleic acids. If the target is mRNA, the sample is obtained
from a tissue in which the mRNA is expressed. If the
15 polynucleotide in the sample is RNA, it is usually reverse
transcribed to DNA. DNA samples or cDNA resulting from
reverse transcription are usually amplified, e.g., by PCR.
Depending on the selection of primers and amplifying
enzyme(s), the amplification product can be RNA or DNA.
20 Paired primers are selected to flank the borders of a target
polynucleotide of interest. More than one target can be
simultaneously amplified by multiplex PCR in which multiple
paired primers are employed. The target can be labelled at
one or more nucleotides during or after amplification. For
25 some target polynucleotides (depending on size of sample),
e.g., episomal DNA, sufficient DNA is present in the tissue
sample to dispense with the amplification step.

When the target strand is prepared in single-stranded
form as in preparation of target RNA, the sense of the strand
30 should of course be complementary to that of the probes on the
chip. This is achieved by appropriate selection of primers.
The target is preferably fragmented before application to the
chip to reduce or eliminate the formation of secondary
structures in the target. The average size of targets
35 segments following hybridization is usually larger than the
size of probe on the chip.

II. ILLUSTRATIVE CHIPS

A. HIV Chip

HIV has infected a large and expanding number of people, resulting in massive health care expenditures. HIV can rapidly become resistant to drugs used to treat the infection, primarily due to the action of the heterodimeric protein (51 kDa and 66 kDa) HIV reverse transcriptase (RT) both subunits of which are encoded by the 1.7 kb *pol* gene. The high error rate (5-10 per round) of the RT protein is believed to account for the hypermutability of HIV. The nucleoside analogues, i.e., AZT, ddI, ddC, and d4T, commonly used to treat HIV infection are converted to nucleotide analogues by sequential phosphorylation in the cytoplasm of infected cells, where incorporation of the analogue into the viral DNA results in termination of viral replication, because the 5' -> 3' phosphodiester linkage cannot be completed. However, after about 6 months to 1 year of treatment or less, HIV typically mutates the RT gene so as to become incapable of incorporating the analogue and so resistant to treatment. Several mutations known to be associated with drug resistance are shown in the table below. After a virus having drug resistance via a mutation becomes predominant, the patient suffers dramatically increased viral load, worsening symptoms (typically more frequent and difficult-to-treat infections), and ultimately death. Switching to a different treatment regimen as soon as a resistant mutant virus takes hold may be an important step in patient management which prolongs patient life and reduces morbidity during life.

TABLE 3
SOME RT MUTATIONS ASSOCIATED WITH DRUG RESISTANCE

ANTIVIRAL	CODON	aa CHANGE	nt CHANGE
AZT	67	Asp -> Asn	GAC -> AAC
AZT	70	Lys -> Arg	AAA -> AGA
AZT	215	Thr -> Phe or Tyr	ACC -> TTC or TAC
AZT	219	Lys -> Gln or Glu	AAA -> CAA or GAA
AZT	41	Met -> Leu	ATG -> TTG or CTG
ddI and ddC	184	Met -> Val	ATG -> GTG
ddI and ddC	74	Leu -> Val	
TIBO 82150	100	Leu -> Ile	
ddC	65	Lys -> Asn	AAA -> AGA
ddC	69	Thr -> Asp	ACT -> GAT
3TC	184	Met -> Val	ATG -> GTG or GTA
3TC	184	Met -> Ile	ATG -> ATA
AZT + ddI	62	Ala -> Val	GCC -> GTC
AZT + ddI	75	Val -> Ile	GTA -> ATA
AZT + ddI	77	Phe -> Leu	TTC -> TTA
AZT + ddI	116	Phe -> Tyn	TTT -> TAT
AZT + ddI	151	Gln -> Met	CAG -> ATG
Nevaripine	103	Lys -> Asn	AAA -> AAT
	106	Val -> Ala	GTA -> GCA
	106		
	184	Tyr -> Cys	TAT -> TGT
	188	Tyr -> His	TAT -> CAT
	190	Gly -> Ala	GGA -> GCA

N.B.: Other mutations confer resistance to other drugs.

A second important therapeutic target for anti-HIV drugs is the aspartyl protease enzyme encoded by the HIV genome, whose function is required for the formation of infectious progeny. See Robbins & Plattner, *J. Acquired Immune Deficiency Syndromes* 6, 162-170 (1993); Kozal et al., *Curr. Op. Infect. Dis.* 7:72-81 (1994). The protease function in

processing of viral precursor polypeptides to their active forms. Drugs targeted against this enzyme do not impair endogenous human proteases, thereby achieving a high degree of selective toxicity. Moreover, the protease is expressed later
5 in the life-cycle than reverse transcriptase, thereby offering the possibility of a combined attack on HIV at two different times in its life-cycle. As for drugs targeted against the reverse transcriptase, administration of drugs to the protease can result in acquisition of drug resistance through mutation
10 of the protease. By monitoring the protease gene from patients, it is possible to detect the occurrence of mutations, and thereby make appropriate adjustments in the drug(s) being administered.

In addition to being infected with HIV, AIDS patients are
15 often also infected with a wide variety of other infectious agents giving rise to a complex series of symptoms. Often diagnosis and treatment is difficult because many different pathogens (some life-threatening, others routine) cause similar symptoms. Some of these infections, so-called
20 opportunistic infections, are caused by bacterial, fungal, protozoan or viral pathogens which are normally present in small quantity in the body, but are held in check by the immune system. When the immune system in AIDS patients fails, these normally latent pathogens can grow and generate rampant
25 infection. In treating such patients, it would be desirable simultaneously to diagnose the presence or absence of a variety of the most lethal common infections, determine the most effective therapeutic regime against the HIV virus, and monitor the overall status of the patient's infection.

30 The present invention provides DNA chips for detecting the multiple mutations in HIV genes associated with resistance to different therapeutics. These DNA chips allow physicians to monitor mutations over time and to change therapeutics if resistance develops. Some chips also provide probes for
35 diagnosis of pathogenic microorganisms that typically occur in AIDS patients.

The sequence selected as a reference sequence can be from anywhere in the HIV genome, but should preferably cover a

region of the HIV genome in which mutations associated with drug resistance are known to occur. A reference sequence is usually between about 5, 10, 20, 50, 100, 500, 1000, 5,000 or 10,000 bases in length, and preferably is about 100-1700 bases in length. Some reference sequences encompass at least part of the reverse transcriptase sequence encoded by the pol gene. Preferably, the reference sequence encompasses all, or substantially all (i.e, about 75 or 90%) of the reverse transcriptase gene. Reverse transcriptase is the target of several drugs and as noted, above, the coding sequence is the site of many mutations associated with drug resistance. In some chips, the reference sequence contains the entire region coding reverse transcriptase (850 bp), and in other chips, subfragments thereof. In some chips, the reference sequence includes other subfragments of the pol gene encoding HIV protease or endonuclease, instead of, or as well as the segment encoding reverse transcriptase. In some chips, the reference sequence also includes other HIV genes such as env or gag as well as or instead of the reverse transcriptase gene. Certain regions of the gag and env genes are relatively well conserved, and their detection provides a means for identifying and quantifying the amount of HIV virus infecting a patient. In some chips, the reference sequence comprises an entire HIV genome.

It is not critical from which strain of HIV the reference sequence is obtained. HIV strains are classified as HIV-I, HIV-II or HIV-III, and within these generic groupings there are several strains and polymorphic variants of each of these. BRU, SF2, HXB2, HXB2R are examples of HIV-1 strains, the sequences of which are available from GenBank. The reverse transcriptase genes of the BRU and SF2 strains differ at 23 nucleotides. The HXB2 and HXB2R strains have the same reverse transcriptase gene sequence, which differs from that of the BRU strain at four nucleotides, and that of SF2 by 27 nucleotides. In some chips, the reference sequence corresponds exactly to the reverse transcriptase sequence in the wildtype version of a strain. In other chips, the reference sequence corresponds to a consensus sequence of

several HIV strains. In some chips, the reference sequence corresponds to a mutant form of a HIV strain.

Chips are designed in accordance with the tiling strategies noted above. The probes are designed to be
5 complementary to either the coding or noncoding strand of the HIV reference sequence. If only one strand is to be read, it is preferable to read the coding strand. The greater percentage of A residues in this strand relative to the
10 noncoding strand generally result in fewer regions of ambiguous sequence.

Some chips contain additional probes or groups of probes designed to be complementary to a second reference sequence. The second reference sequence is often a subsequence of the first reference sequence bearing one or more commonly
15 occurring HIV mutations or interstrain variations (e.g., within codons 67, 70, 215 or 219 of the reverse transcriptase gene). The inclusion of a second group is particularly useful for analyzing short subsequences of the primary reference sequence in which multiple mutations are expected to occur
20 within a short distance commensurate with the length of the probes (i.e., two or more mutations within 9 to 21 bases).

The total number of probes on the chips depends on the tiling strategy, the length of the reference sequence and the options selected with respect to inclusion of multiple probe
25 lengths and secondary groups of probes to provide confirmation of the existence of common mutations. To read much or all of the HIV reverse transcriptase gene (857 b for the BRU strain), chips tiled by the basic strategy typically contain at least $857 \times 4 = 3428$ probes.

30 The target HIV polynucleotide, whose sequence is to be determined, is usually isolated from blood samples (peripheral blood lymphocytes or PBMC) in the form of RNA. The RNA is reverse transcribed to DNA, and the DNA product is then amplified. Depending on the selection of primers and
35 amplifying enzyme, the amplification product can be RNA or DNA. Suitable primers for amplification of target are shown in the table below.

TABLE 4
AMPLIFICATION OF TARGET

TARGET SIZE	FORWARD PRIMER	REVERSE PRIMER
1,742 bp	GTAGAATTCTGTTGACTCAGATTGG	GATAAGCTTGGGCCTTATCTATTCCAT
535 bp	AAATCCATACAATACTCCAGTATTGC	ACCCATCCAAAGGAATGGAGGTTCTTTC
323 bp	Genbank # K02013 1889-1908	bases 2211-2192
	AATTAACCCTCACTAAAGGGGaga ggaagaatctgtgactcagattggt (RT#1-T3)	AATTTAATACGACTCACTATAGGGGAmcccca ctaacctctgtatgcatgaca-3' (89-391 T7)
	AATTAACCCTCACTAAAGGGGaga agtatactgcattaccacctaagta (RT#3-T3)	
	TAATACGACTCACTATAGGGGAGA tcgacgcaggactcggttgcgtgaa (HV1-T2)	
	AATTAACCCTCACTAAAGGGGAGA ccttgtaagtcattggcttaaaaggta (HV2-T3)	

In another aspect of the invention, chips are provided for simultaneous detection of HIV and microorganisms that commonly parasitize AIDS patients (e.g., cytomegalovirus (CMV), Pneumocystis carini (PCP), fungi (candida albicans), mycobacteria). Non-HIV viral pathogens are detected and their drug resistance determined using a similar strategy as for HIV. That is groups of probes are designed to show complementarity to a target sequence from a region of the genome of a nonviral pathogen known to be associated with acquisition of drug resistance. For example, CMV and HSV viruses, which frequently co-parasitize AIDS patients, undergo mutations to acquire resistance to acyclovir.

For detection of non-viral pathogens, the chips include an array of probes which allow full-sequence determination of 16S ribosomal RNA or corresponding genomic DNA of the pathogens. The additional probes are designed by the same principles as described above except that the target sequence is a variable region from a 16S RNA (or corresponding DNA) of a pathogenic microorganism. Alternatively, the target sequence can be a consensus sequences of variable 16S rRNA regions from multiple organisms. 16S ribosomal DNA and RNA is present in all organisms (except viruses) and the sequence of the DNA or RNA is closely related to the evolutionary genetic distance between any two species. Hence, organisms which are quite close in type (e.g., all mycobacteria) share a common

region of 16S rDNA, and differ in other regions (variable regions) of the 16S rRNA. These differences can be exploited to allow identification of the different subtype strains. The full sequence of 16S ribosomal RNA or DNA read from the chip
5 is compared against a database of the sequence of thousands of known pathogens to type unambiguously most nonviral pathogens infecting AIDS patients.

In a further embodiment, the invention provides chips which also contain probes for detection of bacterial genes
10 conferring antibiotic resistance. An antibiotic resistance gene can be detected by hybridization to a single probe employed in a reverse dot blot format. Alternatively, a group of probes can be designed according to the same principles discussed above to read all or part the DNA sequence encoding
15 an antibiotic resistance gene. Analogous probes groups are designed for reading other antibiotic resistance gene sequences. Antibiotic resistance frequently resides in one of the following genes in microorganisms coparasitizing AIDS patients: rpoB (encoding RNA polymerase), katG (encoding
20 catalase peroxidase, and DNA gyrase A and B genes.

The inclusion of probes for combinations of tests on a single chip simulates the clinical diagnosis tree that a physician would follow based on the presentation of a given syndrome which could be caused by any number of possible
25 pathogens. Such chips allow identification of the presence and titer of HIV in a patient, identification of the HIV strain type and drug resistance, identification of opportunistic pathogens, and identification of the drug resistance of such pathogens. Thus, the physician is
30 simultaneously apprised of the full spectrum of pathogens infecting the patient and the most effective treatments therefor.

Exemplary HIV Chips(a) HV 273

The HV 273 chip contains an array of oligonucleotide probes for analysis of an 857 base HIV amplicon between nucleotides 2090 and 2946 (HIVBRU strain numbering). The chip contains four groups of probes: 11 mers, 13 mers, 15 mers and 17 mers. From top to bottom, the HV 273 chip is occupied by rows of 11 mers, followed by rows of 13 mers, followed by rows of 15 mers followed by rows of 17 mers. The interrogation position is nucleotide 6, 7, 8 and 9 respectively in the different sized chips. This arrangement of the different sized probes is referred to as being "in series." Within each size group, there are four probe sets laid down in an A-lane, a C-lane a G-lane and a T-lane respectively. Each lane contains an overlapping series of probes with one probe for each nucleotide in the 2090-2946 HIV reverse transcriptase reference sequence. (i.e., 857 probes per lane). The lanes also include a few column positions which are empty or occupied by control probes. These positions serve to orient the chip, determine background fluorescence and punctuate different subsequences within the target. The chip has an area of 1.28 x 1.28 cm, within which the probes form a 130 X 135 matrix (17,550 cells total). The area occupied by each probe (i.e., a probe cell) is about 98 X 95 microns.

The chip was tested for its capacity to sequence a reverse transcriptase fragment from the HIV strain SF2. An 831 bp RNA fragment (designated pPol19) spanning most of the HIV reverse transcriptase coding sequence was amplified by PCR, using primers tagged with T3 and T7 promoter sequences. The primers, designated RT#1-T3 and 89-391 T7 are shown in Table 4; see also Gingeras et al., *J. Inf. Dis.* 164, 1066-1074 (1991) (incorporated by reference in its entirety for all purposes). RNA was labelled by incorporation of fluorescent nucleotides. The RNA was fragmented by heating and hybridized to the chip for 40 min at 30 degrees. Hybridization signals were quantified by fluorescence imaging.

Taking the best data from the four probes sets at each position in the target sequence, 715 out of 821 bases were

read correctly (87%). (Comparisons are based on the sequence of pPol19 determined by the conventional dideoxy method to be identical to SF2). In general, the longer sized probes yielded more sequence than the shorter probes. Of the 21 positions at which the SF2 and BRU strains diverged within the target, 19 were read correctly.

Many of the short ambiguous regions in the target arise in segments of the target flanking the points at which the SF2 and BRU sequences diverge. These ambiguities arise because in these regions the comparison of hybridization signals is not drawn between perfectly matched and single base mismatch probes but between a single-mismatched probe and three probes having two mismatches. These ambiguities in reading an SF2 sequence would not detract from the chip's ability to read a BRU sequence either alone or in a mixture with an SF2 target sequence.

In a variation of the above procedure, the chip was treated with RNase after hybridization of the pPol19 target to the probes. Addition of RNase digests mismatched target and thereby increases the signal to noise ratio. RNase treatment increased the number of correctly read bases to 743/821 or 90% (combining the data from the four groups of probes).

In a further variation, the RNA target was replaced with a DNA target containing the same segment of the HIV genome. The DNA probe was prepared by linear amplification using Taq polymerase, RT#1-T3 primer, and fluorescein d-UTP label. The DNA probe was fragmented with uracil DNA glycosylase and heat treatment. The hybridization pattern across the array and percentage of readable sequence were similar to those obtained using an RNA target. However, there were a few regions of sequence that could be read from the RNA target that could not be read from the DNA target and vice versa.

(b) HV 407 Chip

The 407 chip was designed according to the same principles as the HV 273 chip, but differs in several respects. First, the oligonucleotide probes on this chip are designed to exhibit perfect sequence identity (with the

exception of the interrogation position on each probe) to the HIV strain SF2 (rather than the BRU strain as was the case for the HV 273 chip). Second, the 407 chip contains 13 mers, 15 mers, 17 mers and 19 mers (with interrogation positions at nucleotide 7, 8, 9 and 10 respectively), rather than the 11 mers, 13 mers, 15 mers and 17 mers on the HV 273 chip. Third, the different sized groups of oligomers are arranged in parallel in place of the in-series arrangement on the HV 273 chip. In the parallel arrangement, the chip contains from top to bottom a row of 13 mers, a row of 15 mers, a row of 17 mers, a row of 19 mers, followed by a further row of 13 mers, a row of 15 mers, a row of 17 mers, a row of 19 mers, followed by a row of 13 mers, and so forth. Each row contains 4 lanes of probes, an A lane, a C lane, a G lane and a T lane, as described above. The probes in each lane tile across the reference sequence. The layout of probes on the HV 407 chip is shown in Fig. 10.

The 407 chip was separately tested for its ability to sequence two targets, pPol19 RNA and 4MUT18 RNA. pPol19 contains an 831 bp fragment from the SF2 reverse transcriptase gene which exhibits perfect complementarity to the probes on the 407 chip (except of course for the interrogation positions in three of the probes in each column). 4MUT18 differs from the reference sequence at thirty-one positions within the target, including five positions in codons 67, 70, 215 and 219 associated with acquisition of drug resistance. Target RNA was prepared, labelled and fragmented as described above and hybridized to the HV 407 chip. The hybridization pattern for the pPol19 target is shown in Fig. 11.

The sequences read off the chip for the pPol19 and 4MUT18 targets are both shown in Fig. 12 (although the two sequences were determined in different experiments). The sequence labelled wildtype in the Figure is the reference sequence. The four lanes of sequence immediately below the reference sequence are the respective sequences read from the four-sized groups of probes for the pPol19 target (from top-to-bottom, 13 mers, 15 mers, 17 mers and 19 mers). The next four lanes of sequence are the sequences read from the four-sized groups of

probes for the 4MUT18 target (from top-to-bottom in the same order). The regions of sequences shown in normal type are those that could be read unambiguously from the chip. Regions where sequence could not be accurately read are shown

5 highlighted. Some regions of sequence that could not be read from one sized set of probes could be read from another.

Taking the best result from the four sized groups of probes at each column position, about 97% of bases in the pPoll9 sequence and about 90% of bases in the 4MUT18 sequence
10 were read accurately. Of the 31 nucleotide differences between 4MUT18 and the reference sequence, twenty-seven were read correctly including three of the nucleotide changes associated with acquisition of drug resistance. Of the ambiguous regions in the 4MUT18 sequence determination, most
15 occurred in the 4MUT18 segments flanking points of divergence between the 4MUT18 and reference sequences. Notably, most of the common mutations in HIV reverse transcriptase associated with drug resistance (see Table 3) occur at sequence positions that can be read from the chip. Thus, most of the commonly
20 occurring mutations can be detected by a chip containing an array of probes based on a single reference sequence.

Comparison of the sequence read of the probes of different sizes is useful in determining the optimum size probe to use for different regions of the target. The
25 strategy of customizing probe length within a single group of probe sets minimizes the total number of probes required to read a particular target sequence. This leaves ample capacity for the chip to include probes to other reference sequences (e.g., 16S RNA for pathogenic microorganisms) as discussed
30 below.

The HV 407 chip has also been tested for its capacity to detect mixtures of different HIV strains. The mixture comprises varying proportions of two target sequences; one a
segment of a reverse transcriptase gene from a wildtype SF2
35 strain, the other a corresponding segment from an SF2 strain bearing a codon 67 mutation. See Fig. 13. The Figure also represents the probes on the chip having an interrogation position for reading the nucleotide in which the mutation

occurs. A single probe in the Figure represents four probes on the chip with the symbol (o) indicating the interrogation position, which differs in each of the four probes. Figure 14 shows the fluorescence intensity for the four 13 mers and the four 15 mers having an interrogation position for reading the nucleotide in the target sequence in which the mutation occurs. As the percentage of mutant target is increase, the fluorescence intensity of the probe exhibiting perfect complementarity to the wildtype target decreases, and the intensity of the probe exhibiting perfect complementarity to the mutant sequence increases. The intensities of the other two probes do not change appreciably. It is concluded that the chip can be used to analyze simultaneously a mixture of strains, and that a strain comprising as little as ten percent of a mixture can be easily detected.

c. Protease Chip

A protease chip was constructed using the basic tiling strategy. The chip comprises four probes tiling across a 382 nucleotide span including 297 nucleotides from the protease coding sequence. The reference sequence was a consensus Clay-B HIV protease sequence. Different probes lengths were employed for tiling different regions of the reference sequence. Probe lengths were 11, 14, 17 and 20 nucleotides with interrogation positions at or adjacent to the center of each probe. Lengths were optimized from prior hybridization data employing a chip having multiple tilings, each with a different probe length.

The chip was hybridized to four different single-stranded DNA protease target sequences (HXB2, SF2, NY5, pPol4mut18). Both sense and antisense strands were sequenced. Data from the chip was compared with that from an ABI sequencer. The overall accuracy from sequencing the four targets is illustrated in the Table 5 below.

Table 5

		ABI		Protease Chip	
		Sense	Antisense	Sense	Antisense
5	No call	0	4	9	4
	Ambiguous	6	14	17	8
	Wrong call	2	3	3	1
	TOTAL	8	21	29	13

10

ABI (sense) - 99.5%

Chip (sense) - 98.1%

15

ABI (antisense) - 98.6%

Chip (antisense) - 99.1%

20 Combining the data from sense and antisense strands, both the chip and the ABI sequencer provided 100% accurate data for all of the sequence from all four clones.

In a further test, the chip was hybridized to protease target sequences from viral isolates obtained from four
 25 patients before and after ddI treatment. The sequence read from the chip is shown in Fig. 15. Several mutations (indicated by arrows) have arisen in the samples obtained posttreatment. Particularly noteworthy was the chip's capacity to read a g/a mutation at nucleotide 207,
 30 notwithstanding the presence of two additional mutations (gt) at adjacent positions.

B. Cystic Fibrosis Chips

A number of years ago, cystic fibrosis, the most common
 35 severe autosomal recessive disorder in humans, was shown to be associated with mutations in a gene thereafter named the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene. The CFTR gene is about 250 kb in size and has 27 exons. Wildtype genomic sequence is available for all exonic regions
 40 and exons/intron boundaries (Zielenski et al., *Genomics* 10, 214-228 (1991). The full-length wildtype cDNA sequence has also been described (see Riordan et al., *Science* 245, 1059-1065 (1989). Over 400 mutations have been mapped (see Tsui et al., *Hu. Mutat.* 1, 197-203 (1992). Many of the more common
 45 mutations are shown in Table 6. The most common cystic

fibrosis mutation is a three-base deletion resulting in the omission of amino acid #508 from the CFTR protein. The frequency of mutations varies widely in populations of different geographic or ethnic origin (see column 4 of Table 6). About 90% of all mutations having phenotypic effects occur in coding regions.

Detection of CFTR mutations is useful in a number of respects. For example, screening of populations can identify asymptomatic heterozygous individuals. Such individuals are at risk of giving rise to affected offspring suffering from CF if they reproduce with other such individuals. *In utero* screening of fetuses is also useful in identifying fetuses bearing 2 CFTR mutations. Identification of such mutations offers the possibility of abortion, or gene therapy. For couples known to be at risk of giving rise to affected progeny, diagnosis can be combined with *in vitro* reproduction procedures to identify an embryo having at least one wildtype CF allele before implantation. Screening children shortly after birth is also of value in identifying those having 2 copies of the defective gene. Early detection allows administration of appropriate treatment (e.g., Pulmozyme Antibiotics, Pertussive Therapy) thereby improving the quality of life and perhaps prolonging the life expectancy of an individual.

The source of target DNA for detecting of CFTR mutations is usually genomic. In adults, samples can conveniently be obtained from blood or mouthwash epithelial cells. In fetuses, samples can be obtained by several conventional techniques such as amniocentesis, chorionic villus sampling or fetal blood sampling. At birth, blood from the amniotic chord is a useful tissue source.

The target DNA is usually amplified by PCR. Some appropriate pairs of primers for amplifying segments of DNA including the sites of known mutations are listed in Tables 5 and 6.

Table 7

OLIGO NUMBER	SEQUENCE
787	TCTCCTTGGATATACTTGTGTGAATCAA
788	TCACCAGATTTCGTAGTCTTTTCATA
5	851 GTCTTGTGTTGAAATTCTCAGGGTAT
769	CTTGTACCAGCTCACTACCTAAT
887	ACCTGAGAAGATAGTAAGCTAGATGAA
888	AACTCCGCCTTCCAGTTGTAT
934	TTAGTTTCTAGGGGTGGAAGATACA
10	935 TTAATGACACTGAAGATCACTGTTCTAT
789	CCATTCCAAGATCCCTGATATTGAA
790	GCACATTTTGCAGGTTTATTAGTA
891	TCATGGGCCATGTGCTTTTCAA
892	ACCTTCCAGCACTACAACTAGAA
15	760 CAAGTGAATCCTGAGCGTGATTT
850	GGTAGTGTGAAGGGTTCATATGCATA
762	GATTACATTAGAAGGAAGATGTGCCTTT
763	ACATGAATGACATTTACAGCAAATGCTT
931	GTGACCATATTGTAATGCATGTAGTGA
20	932 ATGGTGAACATATTTCTCAAGAGGTAA
955	TGT CTC TGT AAA CTG ATG GCT AAC A
884	TCGTATAGAGTTGATTGGATTGAGAA
885	CCATTAACCTAATGTGGTCTCATCACAA
886	CTACCATAATGCTTGGGAGAAATGAA
25	782 TCAAAGAATGGCACCAGTGTGAAA
901	TGCTTAGCTAAAGTTAATGAGTTCAT

OLIGO NUMBER	SEQUENCE
784	AATTGTGAAATTGTCTGCCATTCTTAA
785	GATTCACCTACTGAACACAGTCTAACAA
791	AGGCTTCTCAGTGATCTGTTG
792	GAATCATTTCAGTGGGTATAAGCA
1013	GCCATGGTACCTATATGTCACAGAA
1012	TGCAGAGTAATATGAATTTCTTGAGTACA
766	GGGACTCCAAATATTGCTGTAGTAT
1065	GTACCTGTTGCTCCAGGTATGTT

Other primers can be readily devised from the known genomic and cDNA sequences of CFTR. The selection of primers, of course, depends on the areas of the target sequence that are to be screened. The choice of primers also depends on the strand to be amplified. For some regions of the CFTR gene, it makes little difference to the hybridization signal whether the coding or noncoding strand is used. In other regions, one strand may give better discrimination in hybridization signals between matched and mismatched probes than the other. The upper limit in the length of a segment that can be amplified from one pair of PCR primers is about 50 kb. Thus, for analysis of mutants through all or much of the CFTR gene, it is often desirable to amplify several segments from several paired primers. The different segments may be amplified sequentially or simultaneously by multiplex PCR. Frequently, fifteen or more segments of the CFTR gene are simultaneously amplified by PCR. The primers and amplification conditions are preferably selected to generate DNA targets. An asymmetric labelling strategy incorporating fluorescently labelled dNTPs for random labelling and dUTP for target fragmentation to an average length of less than 60 bases is preferred. The use of dUTP and fragmentation with

uracil N-glycosylase has the added advantage of eliminating carry over between samples.

Mutations in the CFTR gene can be detected by any of the tiling strategies noted above. The block tiling strategy is one particularly useful approach. In this strategy, a group (or block) of probes is used to analyze a short segment of contiguous nucleotides (e.g., 3, 5, 7 or 9) from a CFTR gene centered around the site of a mutation. The probes in a group are sometimes referred to as constituting a block because all probes in the group are usually identical except at their interrogation positions. As noted above, the probes may also differ in the presence of leading or trailing sequences flanking regions of complementary. However, for ease of illustration, it will be assumed that such sequences are not present. As an example, to analyze a segment of five contiguous nucleotides from the CFTR gene, including the site of a mutation (such as one of the mutations in Table 6), a block of probes usually contains at least one wildtype probe and five sets of mutant probes, each having three probes. The wildtype probe has five interrogation positions corresponding to the five nucleotides being analyzed from the reference sequence. However, the identity of the interrogation positions is only apparent when the structure of the wildtype probe is compared with that of the probes in the five mutant probe sets. The first mutant probe set comprises three probes, each being identical to the wildtype probe, except in the first interrogation position, which differs in each of the three mutant probes and the wildtype probe. The second through fifth mutant probe sets are similarly composed except that the differences from the wildtype probe occur in the second through fifth interrogation position respectively. Note that in practice, each set of mutant probes is sometimes laid down on the chip juxtaposed with an associated wildtype probe. In this situation, a block would comprise five wildtype probes, each effectively providing the same information. However, visual inspection and confidence analysis of the chip is facilitated by the largely redundant information provided by five wildtype probes.

After hybridization to labelled target, the relative hybridization signals are read from the probes. Comparison of the intensities of the three probes in the first mutant probe set with that of the wildtype probe indicates the identity of the nucleotide in the target sequence corresponding to the first interrogation position. Comparison of the intensities of the three probes in the second mutant probe set with that of the wildtype probe indicates the identity of the nucleotide in the target sequence corresponding to the second interrogation position, and so forth. Collectively, the relative hybridization intensities indicate the identity of each of the five contiguous nucleotides in the reference sequence.

In a preferred embodiment, a first group (or block) of probes is tiled based on a wildtype reference sequence and a second group is tiled based a mutant version of the wildtype reference sequence. The mutation can be a point mutation, insertion or deletion or any combination of these. The combination of first and second groups of probes facilitates analysis when multiple target sequences are simultaneously applied to the chip, as is the case when a patient being diagnosed is heterozygous for the CFTR allele.

The above strategy is illustrated in Fig. 16, which shows two groups of probes tiled for a wildtype reference sequence and a point mutation thereof. The five mutant probe sets for the wildtype reference sequence are designated wt1-5, and the five mutant probe sets for the mutant reference sequence are designated m1-5. The letter N indicates the interrogation position, which shifts by one position in successive probe sets from the same group. The figure illustrates the hybridization pattern obtained when the chip is hybridized with a homozygous wildtype target sequence comprising nucleotides $n-2$ to $n+2$, where n is the site of a mutation. For the group of probes tiled based on the reference sequence, four probes are compared at each interrogation position. At each position, one of the four probes exhibits a perfect match with the target, and the other three exhibit a single-base mismatch. For the group of probes tiled based on the mutant

reference sequence, again four probes are compared at each interrogation position. At position, n, one probe exhibits a perfect match, and three probes exhibit a single base mismatch. Hybridization to a homozygous mutant yields an analogous pattern, except that the respective hybridization patterns of probes tiled on the wildtype and mutant reference sequences are reversed.

The hybridization pattern is very different when the chip is hybridized with a sample from a patient who is heterozygous for the mutant allele (see Fig. 17). For the group of probes tiled based on the wildtype sequence, at all positions but n, one probe exhibits a perfect match at each interrogation position, and the other three probes exhibit a one base mismatch. At position n, two probes exhibit a perfect match (one for each allele), and the other probes exhibit single-base mismatches. For the group of probes tiled on the mutant sequence, the same result is obtained. Thus, the heterozygote point mutant is easily distinguished from both the homozygous wildtype and mutant forms by the identity of hybridization patterns from the two groups of probes.

Typically, a chip comprises several paired groups of probes, each pair for detecting a particular mutation. For example, some chips contain 5, 10, 20, 40 or 100 paired groups of probes for detecting the corresponding numbers of mutations. Some chips are customized to include paired groups of probes for detecting all mutations common in particular populations (see Table 6). Chips usually also contain control probes for verifying that correct amplification has occurred and that the target is properly labelled.

The goal of the tiling strategy described above is to focus on short regions of the CFTR region flanking the sites of known mutation. Other tiling strategies analyze much larger regions of the CFTR gene, and are appropriate for locating and identifying hitherto uncharacterized mutations. For example, the entire genomic CFTR gene (250 kb) can be tiled by the basic tiling strategy from an array of about one million probes. Synthesis and scanning of such an array of probes is entirely feasible. Other tiling strategies, such as

the block tiling, multiplex tiling or pooling can cover the entire gene with fewer probes. Some tiling strategies analyze some or all of components of the CFTR gene, such as the cDNA coding sequence or individual exons. Analysis of exons 10 and 11 is particularly informative because these are location of many common mutations including the $\Delta F508$ mutation.

Exemplary CFTR chips

One illustrative chip bears an array of 1296 probes covering the full length of exon 10 of the CFTR gene arranged in a 36 x 36 array of 356 μm elements. The probes in the array can have any length, preferably in the range of from 10 to 18 residues and can be used to detect and sequence any single-base substitution and any deletion within the 192-base exon, including the three-base deletion known as $\Delta F508$. As described in detail below, hybridization of nanomolar concentrations of wild-type and $\Delta F508$ oligonucleotide target nucleic acids labeled with fluorescein to these arrays produces highly specific signals (detected with confocal scanning fluorescence microscopy) that permit discrimination between mutant and wild-type target sequences in both homozygous and heterozygous cases.

Sets of probes of a selected length in the range of from 10 to 18 bases and complementary to subsequences of the known wild-type CFTR sequence are synthesized starting at a position a few bases into the intron on the 5'-side of exon 10 and ending a few bases into the intron on the 3'-side. There is a probe for each possible subsequence of the given segment of the gene, and the probes are organized into a "lane" in such a way that traversing the lane from the upper left-hand corner of the chip to the lower righthand corner corresponded to traversing the gene segment base-by-base from the 5'-end. The lane containing that set of probes is, as noted above, called the "wild-type lane."

Relative to the wild-type lane, a "substitution" lane, called the "A-lane", was synthesized on the chip. The A-lane probes were identical in sequence to an adjacent (immediately below the corresponding) wild-type probe but contained, regardless of the sequence of the wild-type probe, a dA

residue at position 7 (counting from the 3'-end). In similar fashion, substitution lanes with replacement bases dC, dG, and dT were placed onto the chip in a "C-lane," a "G-lane," and a "T-lane," respectively. A sixth lane on the chip consisted of probes identical to those in the wild-type lane but for the deletion of the base in position 7 and restoration of the original probe length by addition to the 5'-end the base complementary to the gene at that position.

The four substitution lanes enable one to deduce the sequence of a target exon 10 nucleic acid from the relative intensities with which the target hybridizes to the probes in the various lanes. Various versions of such exon 10 DNA chips were made as described above with probes 15 bases long, as well as chips with probes 10, 14, and 18 bases long. For the results described below, the probes were 15 bases long, and the position of substitution was 7 from the 3'-end.

The sequences of several important probes are shown below. In each case, the letter "X" stands for the interrogation position in a given column set, so each of the sequences actually represents four probes, with A, C, G, and T, respectively, taking the place of the "X." Sets of shorter probes derived from the sets shown below by removing up to five bases from the 5'-end of each probe and sets of longer probes made from this set by adding up to three bases from the exon 10 sequence to the 5'-end of each probe, are also useful and provided by the invention.

```

3'-TTTATAXTAGAAACC
3'- TTATAGXAGAAACCA
3'- TATAGTXGAAACCAC
30 3'- ATAGTAXAAACCACA
3'- TAGTAGXAACCACAA
3'- AGTAGAXACCACAAA
3'- GTAGAAXCCACAAAG
3'- TAGAAAXCACAAAGG
35 3'- AGAAACXACAAAGGA

```

To demonstrate the ability of the chip to distinguish the $\Delta F508$ mutation from the wild-type, two synthetic target

nucleic acids were made. The first, a 39-mer complementary to a subsequence of exon 10 of the CFTR gene having the three bases involved in the $\Delta F508$ mutation near its center, is called the "wild-type" or wt508 target, corresponds to

5 positions 111-149 of the exon, and has the sequence shown below:

5'-CATTAAGAAAATATCATCTTTGGTGTTCCTATGATGA.

The second, a 36-mer probe derived from the wild-type target by removing those same three bases, is called the "mutant" target or mu508 target and has the sequence shown below, first with dashes to indicate the deleted bases, and then without dashes but with one base underlined (to indicate the base detected by the T-lane probe, as discussed below):

5'-CATTAAGAAAATATCAT---TGGTGTTCCTATGATGA;

15 5'-CATTAAGAAAATATCATTTGGTGTTCCTATGATGA.

Both targets were labeled with fluorescein at the 5'-end.

In three separate experiments, the wild-type target, the mutant target, and an equimolar mixture of both targets was exposed (0.1 nM wt508, 0.1 nM mu508, and 0.1 nM wt508 plus 0.1 nM mu508, respectively, in a solution compatible with nucleic acid hybridization) to a CF chip. The hybridization mixture was incubated overnight at room temperature, and then the chip was scanned on a reader (a confocal fluorescence microscope in photon-counting mode); images of the chip were constructed from the photon counts) at several successively higher temperatures while still in contact with the target solution. After each temperature change, the chip was allowed to equilibrate for approximately one-half hour before being scanned. After each set of scans, the chip was exposed to denaturing solvent and conditions to wash, i.e., remove target that had bound, the chip so that the next experiment could be done with a clean chip.

The results of the experiments are shown in Figures 18, 19, 20, and 21. Figure 18, in panels A, B, and C, shows an image made from the region of a DNA chip containing CFTR exon 10 probes; in panel A, the chip was hybridized to a wild-type target; in panel C, the chip was hybridized to a mutant $\Delta F508$ target; and in panel B, the chip was hybridized to a mixture

of the wild-type and mutant targets. Figure 19, in sheets 1 - 3, corresponding to panels A, B, and C of Figure 3, shows graphs of fluorescence intensity versus tiling position. The labels on the horizontal axis show the bases in the wild-type sequence corresponding to the position of substitution in the
5 respective probes. Plotted are the intensities observed from the features (or synthesis sites) containing wild-type probes, the features containing the substitution probes that bound the most target ("called"), and the feature containing the
10 substitution probes that bound the target with the second highest intensity of all the substitution probes ("2nd Highest").

These figures show that, for the wild-type target and the equimolar mixture of targets, the substitution probe with a
15 nucleotide sequence identical to the corresponding wild-type probe bound the most target, allowing for an unambiguous assignment of target sequence as shown by letters near the points on the curve. The target wt508 thus hybridized to the probes in the wild-type lane of the chip, although the
20 strength of the hybridization varied from probe-to-probe, probably due to differences in melting temperature. The sequence of most of the target can thus be read directly from the chip, by inference from the pattern of hybridization in the lanes of substitution probes (if the target hybridizes
25 most intensely to the probe in the A-lane, then one infers that the target has a T in the position of substitution, and so on).

For the mutant target, the sequence could similarly be called on the 3'-side of the deletion. However, the intensity
30 of binding declined precipitously as the point of substitution approached the site of the deletion from the 3'-end of the target, so that the binding intensity on the wild-type probe whose point of substitution corresponds to the T at the 3'-end of the deletion was very close to background. Following that
35 pattern, the wild-type probe whose point of substitution corresponds to the middle base (also a T) of the deletion bound still less target. However, the probe in the T-lane of that column set bound the target very well. Examination of

the sequences of the two targets reveals that the deletion places an A at that position when the sequences are aligned at their 3'-ends and that the T-lane probe is complementary to the mutant target with but two mismatches near an end (shown
5 below in lower-case letters, with the position of substitution underlined):

Target: 5'-CATTAAAGAAAATATCATTGGTGTTCCTATGATGA

Probe: 3'-TagTAGTAACCACAA

Thus the T-lane probe in that column set calls the correct
10 base from the mutant sequence. Note that, in the graph for the equimolar mixture of the two targets, that T-lane probe binds almost as much target as does the A-lane probe in the same column set, whereas in the other column sets, the probes that do not have wild-type sequence do not bind target at all
15 as well. Thus, that one column set, and in particular the T-lane probe within that set, detects the $\Delta F508$ mutation under conditions that simulate the homozygous case and also conditions that simulate the heterozygous case.

Although in this example the sequence could not be
20 reliably deduced near the ends of the target, where there is not enough overlap between target and probe to allow effective hybridization, and around the center of the target, where hybridization was weak for some other reason, perhaps high AT-content, the results show the method and the probes of the
25 invention can be used to detect the mutation of interest. The mutant target gave a pattern of hybridization that was very similar to that of the wt508 target at the ends, where the two share a common sequence, and very different in the middle, where the deletion is located. As one scans the image from
30 right to left, the intensity of hybridization of the target to the probes in the wild-type lane drops off much more rapidly near the center of the image for mu508 than for wt508; in addition, there is one probe in the T-lane that hybridizes intensely with mu508 and hardly at all with wt508. The
35 results from the equimolar mixture of the two targets, which represents the case one would encounter in testing a heterozygous individual for the mutation, are a blend of the results for the separate targets, showing the power of the

invention to distinguish a wild-type target sequence from one containing the Δ F508 mutation and to detect a mixture of the two sequences.

The results above clearly demonstrate how the DNA chips
5 of the invention can be used to detect a deletion mutation, Δ F508; another model system was used to show that the chips can also be used to detect a point mutation as well. One mutation in the CFTR gene is G480C, which involves the replacement of the G in position 46 of exon 10 by a T,
10 resulting in the substitution of a cysteine for the glycine normally in position #480 of the CFTR protein. The model target sequences included the 21-mer probe wt480 to represent the wild-type sequence at positions 37-55 of exon 10:
5'-CCTTCAGAGGGTAAAATTAAG and the 21-mer probe mu480 to
15 represent the mutant sequence:
5'-CCTTCAGAGTGTAATAAATTAAG.

In separate experiments, a DNA chip was hybridized to each of the targets wt480 and mu480, respectively, and then scanned with a confocal microscope. Figure 20, in panels A,
20 B, and C, shows an image made from the region of a DNA chip containing CFTR exon 10 probes; in panel A, the chip was hybridized to the wt480 target; in panel C, the chip was hybridized to the mu480 target; and in panel B, the chip was hybridized to a mixture of the wild-type and mutant targets.
25 Figure 21, in sheets 1 - 3, corresponding to panels A, B, and C of Figure 20, shows graphs of fluorescence intensity versus tiling position. The labels on the horizontal axis show the bases in the wild-type sequence corresponding to the position of substitution in the respective probes. Plotted are the
30 intensities observed from the features (or synthesis sites) containing wild-type probes, the features containing the substitution probes that bound the most target ("called"), and the feature containing the substitution probes that bound the target with the second highest intensity of all the
35 substitution probes ("2nd Highest").

These figures show that the chip could be used to sequence a 16-base stretch from the center of the target wt480 and that discrimination against mismatches is quite good

throughout the sequenced region. When the DNA chip was exposed to the target mu480, only one probe in the portion of the chip shown bound the target well: the probe in the set of probes devoted to identifying the base at position 46 in exon 10 and that has an A in the position of substitution and so is fully complementary to the central portion of the mutant target. All other probes in that region of the chip have at least one mismatch with the mutant target and therefore bind much less of it. In spite of that fact, the sequence of mu480 for several positions to both sides of the mutation can be read from the chip, albeit with much-reduced intensities from those observed with the wild-type target.

The results also show that, when the two targets were mixed together and exposed to the chip, the hybridization pattern observed was a combination of the other two patterns. The wild-type sequence could easily be read from the chip, but the probe that bound the mu480 target so well when only the mu480 target was present also bound it well when both the mutant and wild-type targets were present in a mixture, making the hybridization pattern easily distinguishable from that of the wild-type target alone. These results again show the power of the DNA chips of the invention to detect point mutations in both homo- and heterozygous individuals.

To demonstrate clinical application of the DNA chips of the invention, the chips were used to study and detect mutations in nucleic acids from genomic samples. Genomic samples from a individual carrying only the wild-type gene and an individual heterozygous for $\Delta F508$ were amplified by PCR using exon 10 primers containing the promoter for T7 RNA polymerase. Illustrative primers of the invention are shown below.

Exon Name Sequence

10	CFi9-T7	TAATACGACTCACTATAGGGAGatgacctaataatgatgggttt
10	CFi10c-T7	TAATACGACTCACTATAGGGAGtagtgtgaagggttcatatgc
10	CFi10c-T3	CTCGGAATTAACCCTCACTAAAGGtagtgtgaagggttcatatgc
11	CFi10-T7	TAATACGACTCACTATAGGGAGagcatactaaaagtgactctc
11	CFi11c-T7	TAATACGACTCACTATAGGGAGacatgaatgacatttacagcaa
11	CFi11c-T3	CGGAATTAACCCTCACTAAAGGacatgaatgacatttacagcaa

These primers can be used to amplify exon 10 or exon 11 sequences; in another embodiment, multiplex PCR is employed, using two or more pairs of primers to amplify more than one exon at a time.

5 The product of amplification was then used as a template for the RNA polymerase, with fluoresceinated UTP present to label the RNA product. After sufficient RNA was made, it was fragmented and applied to an exon 10 DNA chip for 15 minutes, after which the chip was washed with hybridization buffer and
10 scanned with the fluorescence microscope. A useful positive control included on many CF exon 10 chips is the 8-mer 3'-CGCCGCCG-5'. Figure 22, in panels A and B, shows an image made from a region of a DNA chip containing CFTR exon 10 probes; in panel A, the chip was hybridized to nucleic acid
15 derived from the genomic DNA of an individual with wild-type $\Delta F508$ sequences; in panel B, the target nucleic acid originated from a heterozygous (with respect to the $\Delta F508$ mutation) individual. Figure 23, in sheets 1 and 2, corresponding to panels A and B of Figure 22, shows graphs of
20 fluorescence intensity versus tiling position.

These figures show that the sequence of the wild-type RNA can be called for most of the bases near the mutation. In the case of the $\Delta F508$ heterozygous carrier, one particular probe, the same one that distinguished so clearly between the
25 wild-type and mutant oligonucleotide targets in the model system described above, in the T-lane binds a large amount of RNA, while the same probe binds little RNA from the wild-type individual. These results show that the DNA chips of the invention are capable of detecting the $\Delta F508$ mutation in a
30 heterozygous carrier.

Further chips were constructed using the block tiling strategy to provide an array of probes for analyzing a CFTR mutation. The array comprised 93 mm x 96 μ m features arranged into eleven columns and four rows (44 total probes). Probes
35 in five of these columns were from four probe sets tiled based on the wildtype CFTR sequence and having interrogation positions corresponding to the site of a mutation and two bases on either side. Five of the remaining columns contained

four sets of probes tiled based on the mutant version of the CFTR sequence. These probe sets also had interrogation positions corresponding to the site of mutation and two nucleotides on either side. The eleventh column contained
5 four cells for control probes.

Fluorescently labeled hybridization targets were prepared by PCR amplification. 100 μ g of genomic DNA, 0.4 μ M of each primer, 50 μ M each dATP, dCTP, dGTP and dUTP (Pharmacia) n 10mM Tris-Cl, pH 8.3, 50 mM KCl, 2.5 mM MgCl₂ and 2 U Taq
10 polymerase (Perkin-Elmer) were cycled 36 times using a Perkin-Elmer 9600 thermocycler and the following times and temperatures: 95°C, 10 sec., 55°C, 10 sec., 72°C, 30 sec. 10 μ l of this reaction product was used as a template in a second, asymmetric PCR reaction. Conditions included 1 μ M
15 asymmetric PCR primer, 50 μ M each dATP, dCTP, TTP, 25 μ M fluorescein-dGTP (DuPont), 10 mM Tris-Cl, pH 9.1, 75 mM KCl, 3.5 mM MgCl₂. The reaction was cycled 5X with the following conditions: 95°C, 10 sec, 60°C, 10 sec, 55°C, 1 min. and 72°C, 1.5 min. This was immediately followed with another 20 cycles
20 using the following conditions: 95°C, 10 sec, 60°C, 10 sec., 72°C, 1.5 min.

Amplification products were fragmented by treating with 2 U of Uracil-N-glycosylase (Gibco) at 30°C for 30 min. followed by heat denaturation at 95°C for 5 min. Finally, the
25 labeled, fragmented PCR product was diluted into hybridization buffer made up of 5 X SSPE and 1 mM Cetyltrimethylammonium Bromide (CTAB). The dilution factor ranged from 10x to 25x with 40 μ l of sample being diluted into 0.4 ml to 1 ml of hybridization solution.

30 Target hybridization was generally carried out with the chip shaking in a small dish containing 500 μ l to 1 ml total volume of hybridization solution. All hybridizations were done at 30°C constant temperature. Alternatively, some hybridizations were carried out with chips enclosed in a
35 plastic package with the 1 cm x 1 cm chip glued facing a 250 μ l fluid chamber. 250-350 μ l of hybridization solution was introduced and mixed using a syringe pump. Temperature was controlled by interfacing the back surface of the package with

a Peltier heating/cooling device. Following hybridization chips were washed with 5X SSPE, 0.1% Triton X-100 at 25°C-30°C prior to fluorescent image generation.

Hybridized, washed DNA chips were scanned for
5 fluorescence using a stage-scanning confocal epifluorescent microscope and 488nm argon ion laser excitation. Emitted light was collected through a band pass filter centered at 530nm. The resulting fluorescence image was spatially reconstructed and intensity data were then analyzed. Features
10 with the peak fluorescence intensity in each column were identified and compared with any signal intensity at the remaining single base mismatch probe sites in the same column. The sequences of the highest intensity features were then compared across all ten columns of each sub-array to determine
15 whether peak intensity scores for the wild type sequence and the mutant sequence were similar or significantly different. These results were used to generate the genotype call of wild type (high intensity signals only in wild type probe columns), mutant (high intensity signals only in the mutant probe
20 columns) or heterozygous (high intensity signals in both the wild type and mutant probe columns).

Figure 24 (panel A) shows an image of the fluorescence signals in arrays designed to detect the G551D(G>A) and Q552X(C>T) CFTR mutations. The hybridization target is an
25 exon 11 amplicon generated from wild type genomic DNA. Wild type hybridization patterns are evident at both locations. No significant fluorescence signal resulted at any of the features with probes complementary to mutant or mismatched sequences. Relative fluorescence intensities were six fold
30 brighter for the perfect matched wildtype features compared with the background signal intensity at mutant and mismatch features. In addition, the sequence at these loci can be confirmed as AGGTC and GTCAA, respectively, where the bold type face indicates the mutation sites. Figure 24 (panel B)
35 shows the same probe array features after hybridization with a fluorescent target generated from DNA heterozygous for the G551D mutation. Both the wild type and mutant probe columns have features with significant fluorescence intensity,

indicating the hybridization of both wild type and mutant CFTR alleles at this site. Only wildtype probes hybridized with any significant fluorescence signal in the Q552X subarray indicating a wild type target sequence. However, an additional feature that did not hybridize in the first experiment shows significant fluorescence intensity in this experiment. Because the G551D and Q552X mutations are only two bases apart, the a probe sequence in the additional feature has a perfectly matched 12-mer overlap with the mutant G551D target.

Figure 25 (panels A and B) illustrates mutation analysis for $\Delta F508$, a three base pair deletion in Exon 10 of the CFTR gene. In contrast to the hybridization pattern seen in base change mutations, in mutations where bases are inserted or deleted, probe arrays show a different hybridization pattern. Identical probes are synthesized in the two central columns of base substitution arrays. As a result, either mutant or wild type target hybridizations always result in two side-by-side features (a doublet) with high fluorescence intensity at the center of the array. In a heterozygote hybridization, two sets of doublets, one matched to the wild type sequence and one to the mutant sequence occur (Figure 24, panel B). In contrast, wild type and mutant probe column sequences are offset from each other for deletion or insertion mutations and hybridization doublets are not seen. Instead of the six high intensity signals with one doublet, five independent features in alternating columns characterize a homozygote and ten features, one in each column will be positive with heterozygote targets. This is evident from the $\Delta F508$ hybridization pattern in Figure 25, panel A. Although a wildtype target has been hybridized and the highest intensity features confirm the wild type sequence (ATCTT), there is an additional hybridization in the first mutant column. Analysis of that probe sequence shows a 10 base perfect match with the mutant sequence.

The image in Figure 25, panel B resulted from hybridizing a DNA chip with a target homozygous for $\Delta F508$. In this image five features, all with probe sequences

complementary to the mutant show significant signal. The mutation sequence bridging the deletion site, ATTGG, is confirmed. Similar to what was seen in the example of the G551D mutation, there is added information in neighboring subarrays designed to detect the Δ I507 and F508C mutations. This is expected since they are in such close proximity to Δ F508 that their probe sets significantly overlap the Δ F508 probes. The Δ F508 homozygous target has no perfect matches with wild type or mutant probes in the Δ I507 and F508C subarrays. However, there are some low intensity signals within these two blocks of probes. The F508C array has a doublet that matches 11 bases of the mutant Δ F508 target. Similarly, the hybridization in the eighth column of the Δ I507 array has a probe that matches 13/14 bases with the target.

Figure 26 shows hybridization of a heterozygous double mutant Δ F508/F508C to the same array as described above. Conventional reverse dot blot would score this sample as a homozygous Δ F508 mutant. In the present assays, the Δ F508 and F508C alleles are separately detected by the respective subarrays designed to detect these mutations.

C. Chips for Cancer Diagnosis

There are at least two types of genes which are often altered in cancerous cells. The first type of gene is an oncogene such as a mismatch-repair gene, and the second type of gene is a tumor suppressor gene such as a transcription factor. Examples of mismatch repair oncogenes include hMSH2 (Fishel et al., *Cell* 75, 1027-1038 (1993)) and hMLH1 (Papadopoulos et al., *Science* 263, 1625-1628 (1994)). The most well-known example of a tumor suppressor gene is the p53 protein gene (Buchman et al., *Gene* 70, 245-252 (1988)). By monitoring the state of both oncogenes and tumor suppressor genes (individually and in combination) in a patient, it is possible to determine individual susceptibility to a cancer, a patient's prognosis upon cancer diagnosis, and to target therapy more efficiently.

The p53 gene spans 20 kbp in humans and has 11 exons, 10 of which are protein coding (see Tominaga et al., 1992,

Critical Reviews in Oncogenesis 3:257-282, incorporated herein by reference). The gene produces a 53 kilodalton phosphoprotein that regulates DNA replication. The protein acts to halt replication at the G1/S boundary in the cell cycle and is believed to act as a "molecular policeman," shutting down replication when the DNA is damaged or blocking the reproduction of DNA viruses (see Lane, 1992, *Nature* 358:15-16, incorporated herein by reference). The p53 transcription factor is part of a fundamental pathway which controls cell growth. Wild-type p53 can halt cell growth, or in some cases bring about programmed cell death (apoptosis). Such tumor-suppressive effects are absent in a variety of known p53 gene mutations. Moreover, p53 mutants not only deprive a cell of wild-type p53 tumor suppression, they also may spur abnormal cell growth.

In tumor cells, p53 is the most commonly mutated gene discovered to date (see Levine et al., 1991, *Nature* 351:453-456, and Hollstein et al., 1991, *Science* 253:49-53, each of which is incorporated herein by reference). Over half of the 6.5 million patients diagnosed with cancer annually possess p53 mutations in their tumor cells. Among common tumors, about 70% of colorectal cancers, 50% of lung cancers and 40% of breast cancers contain p53 mutations. In all, over 51 types of human tumors have been documented to possess p53 mutations, including bladder, brain, breast, cervix, colon, esophagus, larynx, liver, lung, ovary, pancreas, prostate, skin, stomach, and thyroid tumors (Culotta & Koshland, *Science* 262, 1958-1961 (1993); Rodrigues et al., 1990, *PNAS* 87:7555-7559, incorporated herein by reference). According to data presented by David Sidransky (1992 San Diego Conference), over 400 mutations in p53 are known. The presence of a p53 mutation in a tumor has also been correlated with a patient's prognosis. Patients who possess p53 mutations have a lower 5-year survival rate.

Proper diagnosis of the form of p53 in tumor cells is critical to clinicians to prescribe appropriate therapeutic regimens. For instance, patients with breast cancer who show no invasion of nearby lymph nodes generally do not relapse

after standard surgical treatment and chemotherapy. Of the 25% who do relapse after surgery and chemotherapy, additional chemotherapy is appropriate. At present, there is no clear way to determine which patients will benefit from such
5 additional chemotherapy prior to relapse. However, correlating p53 mutations to tumorigenicity and metastasis provides clinicians with a means to determine whether such additional treatments are warranted.

In addition to facilitating conventional chemotherapy,
10 appropriate diagnosis of p53 mutations provides clinicians with the ability to identify individuals who will benefit the most from gene therapy techniques, in which appropriately operative p53 copies are restored to a tumor site. Clinical p53 gene therapy trials are presently underway (Culotta &
15 Koshland, *supra*).

The analysis of p53 mutations can also be used to identify which carcinogens lead to particular tumors (Harris, *Science* 262, 1980-1981 (1993)). For instance, dietary aflatoxin B₁ exposure is associated with G:C to T:A
20 transversions at residue 249 of p53 in hepatocellular carcinomas (Hsu et al., *Nature* 350, 427 (1991); Bressac et al., *Nature* 350, 429 (1991); Harris, *supra*).

While most described p53 mutations are somatic in origin, some types of cancer are associated with germline p53
25 mutation. For instance, Li-Fraumeni syndrome is a hereditary condition in which individuals receive mutant p53 alleles, resulting in the early onset of various cancers (Harris, *supra*); Frebourg et al., *PNAS* 89, 6413-6417 (1992); Malkin et al., *Science* 250, 1233 (1990)). These mutations are
30 associated with instability in the rest of the genome, creating multiple genetic alterations, and eventually leading to cancer.

hMLH1 and hMSH2 are mismatch repair genes which are causal agents in hereditary nonpolyposis colorectal cancer in
35 individuals with mutant hMLH1 or hMSH2 alleles (Fishel et al., *supra*, and Papadopoulos et al., *supra*). Hereditary nonpolyposis colorectal cancer is a common genetic disorders, affecting about 1 in 200 individuals (Lynch et al.,

Gastroenterology 104, 1535 (1993)). Detection of hMLH1 and hMSH2 mutations in the population allows diagnosis of nonpolyposis colorectal cancer prone individuals prior to the manifestation of disease. This allows for the implementation of special screening programs for cancer-prone individuals to ensure early detection of cancer, thereby enhancing survival rates of afflicted individuals. In addition, genetic counselors may use the information derived from hMLH1 and hMSH2 chips to improve family planning as described for cystic fibrosis chips. The detection of mutations in hMLH1 and hMSH2 individually or in combination with p53 can also be used by clinicians to assess cancer prognosis and treatment modality. Finally, the information can be used to target appropriate individuals for gene therapy.

The entire hMLH1 gene is less than 85 kbp in length, comprising 2268 coding nucleotides (Papadopoulos et al., *supra*). Sequences from the gene have been deposited with GenBank (accession number U07418). Mutations associated with hereditary nonpolyposis colorectal cancer include the deletion of exon 5 (codons 578-632), a 4 base pair deletion of codons 727 and 728 resulting in a shift in the reading frame of the gene, a 4 base pair insertion at codons 755 and 756 resulting in an extension of the COOH terminus, a 371 base pair deletion and frameshift mutation at position 347, and a transversion causing an alteration of codon 252 resulting in the insertion of a stop codon (*id.*).

hMSH2 is a human homologue of the bacterial *MutS* and *S. cerevisiae* MSH mismatch-repair genes. MSH2, like hMLH1 is associated with hereditary nonpolyposis cancer. Although only a few MSH2 gene samples from tumor tissue have been characterized, at least some tumor samples show a T to C transition mutation at position 2020 of the cDNA sequence, resulting in the loss of an intron-exon splice acceptor site.

In view of the role of mutations in p53, MSH2 and/or hMLH1 in hereditary predisposition to cancer, to neoplastic transformation events leading to cancer and to cancer prognosis, it is important to screen individuals to determine whether they possess mutant alleles, and to identify precisely

which mutations the individuals possess. Because many mutations are point mutations, or extremely small insertions or deletions, which are generally undetectable by standard Southern analysis, accurate diagnosis requires a capacity to
5 examine a gene nucleotide-by-nucleotide.

Mutations in the hMSH2, hMLH1 or p53 genes, irrespective of whether previously characterized, can be detected by any of the tiling strategies noted above. Reference sequences of interest include full-length genomic and cDNA sequences of
10 each of these genes and subsequences thereof, such as exons and introns. For example, each nucleotide in the 20 kb p53 genomic sequence can be tiled using the basic strategy with an array of about 80,000 probes. As in the CFTR chip, some reference sequences are comparatively short sequences
15 including the site of a known mutation and a few flanking nucleotides. Some chips tile reference sequences that encompass mutational "hot spots." For instance, a variety of cellular and oncoviral proteins bind to specific regions of p53, including Mdm2, SV40 T antigen, Elb from adenovirus and
20 E6 from human papilloma virus. These binding sites correlate to some extent with observed high frequency somatic mutation regions of p53 found in tumor cells from cancer patients (see Harris et al., *supra*). Hot spots include exons 2, 3, 5, 6, 7 and 8 and the intronic regions between exons 2 and 3, 3 and 4
25 and 4 and 5. Fragments of the hMLH1 gene of particular interest include those encoding codons 578-632, 727, 728, 347, 252. Some chips are tiled to read mutations in each of the hMSH2, hMLH1 and p53 genes, both wildtype and mutant versions.

Standard or asymmetric PCR can be used to generate the
30 target DNA used in the tiling assays described above. In general, PCR is used to amplify hMSH2, hMLH1 or p53 sequences from a tissue of interest such as a tumor. Mixed PCR reactions can also be used to generate hMSH2, hMLH1 or p53
sequences simultaneously in a single reaction mixture. Any of
35 the coding or noncoding sequences from the genes may be amplified for use in the block tiling assays described above.

Table 8 below provides examples of primers which are useful in synthesizing specific regions of hMSH2, hMLH1 and

p53. Other primers can readily be devised from the known genomic and cDNA sequences of the genes. The primers described in Table 8 specific for p53 amplification have ends tailored to facilitate cloning into standard restriction enzyme cloning sites.

Table 8: Examples of PCR primers useful in amplifying regions of p53, hMHH1 and hMSH2.

	Region Amplified	Primer Sequence	Description
10	Exon 5 (p53)	TAA TAC GAC TCA CTA TAG GGA GA CCC TGG GCA ACC AGC CCT GTC GT	Exon 5 T7 Primer (5' T7 to p53 3').
	Exon 5 (p53)	ATG CAA TTA ACC CTC ACT AAA GGG AGA CAC TTG TGC CCT GAC TTT CAA C	Exon 5 T3 Primer (5' T3 to p53 3').
15	Exon 6 (p53)	TAA TAC GAC TCA CTA TAG GGA GCC TCC TCC CAG AGA CCC	Exon 6 T7 Primer (5' T7 to p53 3').
	Exon 6 (p53)	ATG CAA TTA ACC CTC ACT AA GGG AGA TCC CCA GGC CTC TGA TTC CTC ACT G	Exon 6 T3 Primer (5' T3 to p53 3').
20	Exon 7 (p53)	TAA TAC GAC TCA CTA TAG GGA CTG GGG CAC AGC CAG GCC AGT GTG CA	Exon 7 T7 Primer (5' T7 to p53 3').
	Exon 7 (p53)	ATG CAA TTA ACC CTC ACT AAA GGG AGA GTC TCC CCA AGG CGC ACT GGC CTC A	Exon 7 T3 Primer (5' T3 to p53 3').
	Exon 8 (p53)	TAA TAC GAC TCA CTA TAG GGA GGG CAT AAC TGC ACC CTT GGT CTC CTC C	Exon 8 T7 Primer (5' T7 to p53 3').
25	Exon 8 (p53)	ATG CAA TTA ACC CTC ACT AAA GGG AGA GGA CCT GAT TTC CTT ACT GCC TCT TGC	Exon 8 T3 Primer (5' T3 to p53 3').
	hMSH2	GAC ATG GCG GTG CAG CCG AAG GAG A	Primer for MSH2, 5' to 3'. If used with MSH2 primer below, a 3033 base pair amplicon will result
	hMSH2	CTA TGT CAA TTG CAA ACA GTG CTC AGT TAC AG	Primer for hMSH2 5'to 3'.
	hMLH1	CTT GGC TCT TCT GGC GCC AAA ATG TCG TTC	Primer for hMLH1, 5'to 3'. If used with hMLH1 primer below, a 2484 base pair amplicon will result.
30	hMLH1	TAT GTT AAG ACA CAT CTA TTT ATT TAT AAT CAA TCC	Primer for hMLH1 5' to 3'.

After PCR amplification of the target amplicon one strand of the amplicon can be isolated, i.e., using a biotinylated primer that allows capture of the undesired strand on streptavidin beads. Alternatively, asymmetric PCR can be used to generate a single-stranded target. Another approach involves the generation of single stranded RNA from the PCR product by incorporating a T7 or other RNA polymerase promoter in one of the primers. The single-stranded material can optionally be fragmented to generate smaller nucleic acids with less significant secondary structure than longer nucleic acids.

In one such method, fragmentation is combined with labeling. To illustrate, degenerate 8-mers or other degenerate short oligonucleotides are hybridized to the single-stranded target material. In the next step, a DNA polymerase is added with the four different dideoxynucleotides, each labeled with a different fluorophore. Fluorophore-labeled dideoxynucleotide are available from a variety of commercial suppliers. Hybridized 8-mers are extended by a labeled dideoxynucleotide. After an optional purification step, i.e., with a size exclusion column, the labeled 9-mers are hybridized to the chip. Other methods of target fragmentation can be employed. The single-stranded DNA can be fragmented by partial degradation with a DNase or partial depurination with acid. Labeling can be accomplished in a separate step, i.e., fluorophore-labeled nucleotides are incorporated before the fragmentation step or a DNA binding fluorophore, such as ethidium homodimer, is attached to the target after fragmentation.

30

Exemplary Chips

a. Exon VI Chip

To illustrate the value of the DNA chips of the present invention in such a method, a DNA chip was synthesized by the VLSIPS™ method to provide an array of overlapping probes which represent or tile across a 60 base region of exon 6 of the p53 gene. To demonstrate the ability to detect substitution mutations in the target, twelve different single substitution

mutations (wild type and three different substitutions at each of three positions) were represented on the chip along with the wild type. Each of these mutations was represented by a series of twelve 12-mer oligonucleotide probes, which were
5 complementary to the wild type target except at the one substituted base. Each of the twelve probes was complementary to a different region of the target and contained the mutated base at a different position, e.g., if the substitution was at base 32, the set of probes would be complementary--with the
10 exception of base 32--to regions of the target 21-32, 22-33, and 32-43). This enabled investigation of the effect of the substitution position within the probe. The alignment of some of the probes with a 12-mer model target nucleic acid is shown in Figure 27.

15 To demonstrate the effect of probe length, an additional series of ten 10-mer probes was included for each mutation (see Figure 28). In the vicinity of the substituted positions, the wild-type sequence was represented by every possible overlapping 12-mer and 10-mer probe. To simplify
20 comparisons, the probes corresponding to each varied position were arranged on the chip in the rectangular regions with the following structure: each row of cells represents one substitution, with the top row representing the wild type. Each column contains probes complementary to the same region
25 of the target, with probes complementary to the 3'-end of the target on the left and probes complementary to the 5'-end of the target on the right. The difference between two adjacent columns is a single base shift in the positioning of the probes. Whenever possible, the series of 10-mer probes were
30 placed in four rows immediately underneath and aligned with the 4 rows of 12-mer probes for the same mutation.

To provide model targets, 5' fluoresceinated 12-mers
containing all possible substitutions in the first position of codon 192 were synthesized (see the starred position in the
35 target in Figure 27). Solutions containing 10 nM target DNA in 6X SSPE, 0.25% Triton X-100 were hybridized to the chip at room temperature for several hours. While target nucleic was hybridized to the chip, the fluorophores on the chip were

excited by light from an argon laser, and the chip was scanned with an autofocusing confocal microscope. The emitted signals were processed by a PC to produce an image using image analysis software. By 1 to 3 hours, the signal had reached a plateau; to remove the hybridized target and allow hybridization to another target, the chip was stripped with 60% formamide, 2 X SSPE at 17 °C for 5 minutes. The washing buffer and temperature can vary, but the buffer typically contains 2-to-3X SSPE, 10-to-60% formamide (one can use multiple washes, increasing the formamide concentration by 10% each wash, and scanning between washes to determine when the wash is complete), and optionally a small percentage of Triton X-100, and the temperature is typically in the range of 15-to-18°C.

Very distinct patterns were observed after hybridization with targets with 1 base substitutions and visualization with a confocal microscope and software analysis, as shown in Figure 29. In general, the probes which form perfect matches with the target retain the highest signal. For example, in the first image, the 12-mer probes that form perfect matches with the wild-type (WT) target are in the first row (top). The 12-mer probes with single base mismatches are located in the second, third, and fourth rows and have much lower signals. The data is also depicted graphically in Figure 30. On each graph, the X ordinate is the position of the probe in its row on the chip, and the Y ordinate is the signal at that probe site after hybridization. When a target with a different one base substitution is hybridized the complementary set of probes has the highest signal (see pictures 2, 3, and 4 in Figure 29 and graphs 2, 3, and 4 in Figure 30). In each case, the probe set with no mismatches with the target has the highest signals. Within a 12-mer probe set, the signal was highest at position 6 or 7. The graphs show that the signal difference between 12-mer probes at the same X ordinate tended to be greatest at positions 5 and 8 when the target and the complementary probes formed 10 base pairs and 11 base pairs, respectively. Because tumors often have both WT and mutant p53 genes, mixed target

populations were also hybridized to the chip, as shown in Figure 31. When the hybridization solution consisted of a 1:1 mixture of WT 12-mer and a 12-mer with a substitution in position 7 of the target, the sets of probes that were perfectly matched to both targets showed higher signals than the other probe sets.

The hybridization efficiency of a 10-mer probe array as compared to a 12-mer probe array was also compared. The 10-mer and 12-mer probe arrays gave comparable signals (see graphs 1-4 in Figure 30 and graphs 1-4 in Figure 32). However, the 10-mer probe sets, which are in rows 5-8 (see images in Figure 29), seemed to be better in this model system than the 12-mer probe sets at resolving one target from another, consistent with the expectation that one base mismatches are more destabilizing for 10-mers than 12-mers. Hybridization results within probe sets perfectly matched to target also followed the expectation that, the more matches the individual probe formed with the target, the higher the signal. However, duplexes with two 3' dangles (see Figure 30, position 6 in graphs 1-4) have about as much signal as the probes which are matched along their entire length (see Figure 30, position 7, in graphs 1-4).

This illustrative model system shows that 12-mer targets that differ by one base substitutions can be readily distinguished from one another by the novel probe array provided by the invention and that resolution of the different 12-mer targets was somewhat better with the 10-mer probe sets than with the 12-mer probe sets.

b. Exon V Chip

To analyze DNA from exon 5 of the p53 tumor suppressor gene, a set of overlapping 17-mer probes was synthesized on a chip. The probes for the WT allele were synthesized so as to tile across the entire exon with single base overlaps between probes. For each WT probe, a sets of 4 additional probes, one for each possible base substitution at position 7, were synthesized and placed in a column relative to the WT probe. Exon 5 DNA was amplified by PCR with primers flanking the exon. One of the primers was labeled with fluorescein; the

other primer was labeled with biotin. After amplification, the biotinylated strand was removed by binding to streptavidin beads. The fluoresceinated strand was used in hybridization.

5 About 1/3 of the amplified, single-stranded nucleic acid
was hybridized overnight in 5 X SSPE at 60°C to the probe chip
(under a cover slip). After washing with 6 X SSPE, the chip
was scanned using confocal microscopy. Figure 33 shows an
image of the p53 chip hybridized to the target DNA. Analysis
10 of the intensity data showed that 93.5% of the 184 bases of
exon 5 were called in agreement with the WT sequence (see
Buchman *et al.*, 1988, *Gene* 70: 245-252, incorporated herein by
reference). The miscalled bases were from positions where
probe signal intensities were tied (1.6%) and where non-WT
15 probes had the highest signal intensity (4.9%). Figure 34
illustrates how the actual sequence was read. Gaps in the
sequence of letters in the WT rows correspond to control
probes or sites. Positions at which bases are miscalled are
represented by letters in italic type in cells corresponding
20 to probes in which the WT bases have been substituted by other
bases.

As the diagram indicates, the miscalled bases are from
the low intensity areas of the image, which may be due to
secondary structure in the target or probes preventing
25 intermolecular hybridization. To diminish the effects due to
secondary structure, one can employ shorter targets (i.e., by
target fragmentation) or use more stringent hybridization
conditions. In addition, the use of a set of probes
synthesized by tiling across the other strand of a duplex
30 target can also provide sequence information buried in
secondary structure in the other strand. It should be
appreciated, however, that the pattern of low intensity areas
that forms as a result of secondary structure in the target
itself provides a means to identify that a specific target
35 sequence is present in a sample. Other factors that may
contribute to lower signal intensities include differences in
probe densities and hybridization stabilities.

These results demonstrate the advantages provided by the DNA chips of the invention to genetic analysis. As another example, heterozygous mutations are currently sequenced by an arduous process involving cloning and repurification of DNA.

5 The cloning step is required, because the gel sequencing systems are poor at resolving even a 1:1 mixture of DNA. First, the target DNA is amplified by PCR with primers allowing easy ligation into a vector, which is taken up by transformation of E. coli, which in turn must be cultured, 10 typically on plates overnight. After growth of the bacteria, DNA is purified in a procedure that typically takes about 2 hours; then, the sequencing reactions are performed, which takes at least another hour, and the samples are run on the gel for several hours, the duration depending on the length of 15 the fragment to be sequenced. By contrast, the present invention provides direct analysis of the PCR amplified material after brief transcription and fragmentation steps, saving days of time and labor.

20 D. Mitochondrial Genome Chips

A human cell may have several hundred mitochondria, each with more than one copy of mtDNA. There is strand asymmetry in the base compositions, with one strand (Heavy) being relatively G rich, and the other strand (Light) being C rich. 25 The L strand is 30.9% A, 31.2% C, 13.1% G, and 24.7% T. Human mtDNA is information-rich, encoding some 22 tRNAs, 12S and 16S rRNAs, and 13 polypeptides involved in oxidative phosphorylation. No introns have been detected. RNAs are processed by cleavage at tRNA sequences, and polyadenylated 30 postranscriptionally. In some transcripts, polyadenylation also creates the stop codon, illustrating the parsimony of coding. In many individuals, mtDNA can be treated as haploid. However, some individuals are heteroplasmic (have more than one mtDNA sequence), and the degree of heteroplasmy can vary 35 from tissue to tissue. Also, the rate of replication of mtDNAs can differ and together with random segregation during cell division, can lead to changes in heteroplasmy over time.

The human mitochondrial genome is 16,569 nucleotides

long. The sequence of the L-strand is numbered arbitrarily from the MboI-5/7 boundary in the D-loop region. The complete sequence of the human mitochondrial genome has been published. See Anderson et al., *Nature* 290, 457-465 (1981).

- 5 Mitochondrial DNA is maternally inherited, and has a mutation rate estimated to be tenfold higher than single copy nuclear DNA (Brown et al., *Proc. Natl. Acad. Sci. USA* 76, 1967-1971 (1979)). Human mtDNAs differ, on average, by about 70 base substitutions (Wallace, *Ann. Rev. Biochem.* 61, 1175-1212
10 (1992)). Over 80% of substitutions are transitions (i.e., pyrimidine-pyrimidine or purine-purine).

Analysis of mitochondrial DNA serves several purposes. Detection of mutations in the mitochondrial genome allows diagnosis of a number of diseases. The mitochondrial genome
15 has been identified as the locus of several mutations associated with human diseases. Some of the mutations result in stop codons in structural genes. Such mutations have been mapped and associated with diseases, such as Leber's hereditary optic neuropathy, neurogenic muscular weakness,
20 ataxia and retinitis pigmentosa. Other mutations (nucleotide substitutions) occur in tRNA coding sequences, and presumably cause conformational defects in transcribed tRNA molecules. Such mutations have also been mapped and associated with diseases such as Myoclonic Epilepsy and Ragged Red Fiber
25 Disease. Another type of mutation commonly found is deletions and/or insertions. Some deletions span segments of several kb. Again, such mutations have been mapped and associated with diseases, for example, ocular myopathy and Person Syndrome. See Wallace, *Ann. Rev. Biochem.* 61-1175-1212 (1992)
30 (incorporated by reference in its entirety for all purposes). Early detection of such diseases allows metabolic or genetic therapy to be administered before irretrievable damage has occurred. *Id.* Analysis of mitochondrial DNA is also important for forensic screening. Because the mitochondrial
35 genome is a locus of high variability between individuals, sequencing a substantial length of mitochondrial DNA provides a fingerprint that is highly specific to an individual.

Analysis of mitochondrial DNA is also important for evolutionary and epidemiological studies.

The reference sequence can be an entire mitochondrial genome or any fragment thereof. For forensic and
5 epidemiological studies, the reference sequence is often all or part of the D-loop region in which variability between individuals is greatest (e.g., from 16024-16401 and 29-408). For detection of mutations, analysis of the entire genome is useful as a reference sequence, but shorter segments including
10 the sites of known mutations, and about 1-20 flanking bases are also useful. Some chips have probes tiling paired reference sequences, representing wildtype and mutant versions of a sequence. Tiling a second reference sequence is particularly useful for detecting an insertion mutation
15 occurring in 30-50% of ocular myopathy and Pearson syndrome patients, which consists of direct repeats of the sequence ACCTCCCTCACCA. Some chips include reference sequences from more than one mitochondrial genome.

Mitochondrial reference sequences can be tiled using any
20 of the strategies noted above. The block tiling strategy is particularly useful for analyzing short reference sequences or known mutations. Either the block strategy or the basic strategy is suitable for analyzing long reference sequences. In many of the tiling strategies, it is possible to use fewer
25 probes compared with the number used in other chips without significant loss of sequence information. As noted above, most point mutations in mitochondrial DNA are transitions, so for each wildtype nucleotide in a reference sequence, one of the three possible nucleotide substitutions is much more
30 likely than the other two. Accordingly, in the basic tiling strategy, for example, a reference sequence can be tiled using only two probe sets. One probe sets comprises a plurality of probes, each probe having a segment exactly complementary to the reference sequence. The second probe set comprises a
35 corresponding probe for each probe in the first set. However, a probe from the second probe set differs from the corresponding probe from the first probe set in an interrogation position, in which the probe from the second

probe set includes the transition of the nucleotide present in that position in the probe from the first probe set.

Target mitochondrial DNA can be amplified, labelled and fragmented prior to hybridization using the same procedures as described for other chips. Use of at least two labelled nucleotides is desirable to achieve uniform labelling. Some exemplary primers are described below and other primers can be designed from the known sequence of mitochondrial DNA. Because mitochondrial DNA is present in multiple copies per cell, it can also be hybridized directly to a chip without prior amplification.

Exemplary Chips

The invention provides a DNA chip for analyzing sequences contained in a 1.3 kb fragment of human mitochondrial DNA from the "D-loop" region, the most polymorphic region of human mitochondrial DNA. One such chip comprises a set of 269 overlapping oligonucleotide probes of varying length in the range of 9-14 nucleotides with varying overlaps arranged in 600 x 600 micron features or synthesis sites in an array 1 cm x 1 cm in size. The probes on the chip are shown in columnar form below. An illustrative mitochondrial DNA chip of the invention comprises the following probes (X, Y coordinates are shown, followed by the sequence; "DL3" represents the 3'-end of the probe, which is covalently attached to the chip surface.)

0	0	DL3AGTGGGGTATTT	1	1	DL3GGTTGGTTTGGG
1	0	DL3GGGTATTTAGTT	2	1	DL3TGGGGTTTCTAG
2	0	DL3TTAGTTTATCCAA	3	1	DL3GTTTCTAGTGGG
30	3	0 DL3ATCCAAACCAGG	4	1	DL3AGTGGGGGGTGT
4	0	DL3ACCAGGATCGGA	5	1	DL3GGGGTGTCAAAT
5	0	DL3CGTGTGTGTGTGG	6	1	DL3GTCAAATACATCG
6	0	DL3CGTGTGTGTGTGGC	7	1	DL3ACATCGAATGGAG
7	0	DL3TCGTGTGTGTGTGG	8	1	DL3CGAATGGAGGAG
35	8	0 DL3GTAGGATGGGTC	9	1	DL3GAGGAGTTTCGT
9	0	DL3AGGATGGGTCGT	10	1	DL3TTTCGTTATGTGA
10	0	DL3GATGGGTGCTGT	11	1	DL3ATGTGACTTTTAC
11	0	DL3TGGCGACGATTG	12	1	DL3GACTTTTACAAAT
12	0	DL3GCGACGATTGGG	13	1	DL3AAATCTGCCCCGA
40	13	0 DL3TGGGGGGGA	14	1	DL3AATCTGCCCCGAG
14	0	DL3GAGGGGGGCG	15	1	DL3CCCGAGTGTAAGT
15	0	DL3GGAGGGGGCGA	16	1	DL3AGTGTAGTGGGG
16	0	DL3GAGGGGGCGA	0	2	DL3GGGAGGGTGAG
0	1	DL3GGCTTGTTGG	1	2	DL3GGTGAGGGTATG

2	2	DL3GGTATGATGATTAG	8	5	DL3ATTGTTAAACTTA
3	2	DL3GATTAGAGTAAGT	9	5	DL3AAACTTACAGACG
4	2	DL3TTAGAGTAAGTTA	10	5	DL3ACAGACGTGTCTG
5	2	DL3AAGTTATGTTGGG	11	5	DL3GTGTCGGTGAAA
5	6	DL3GTTGGGGGCG	12	5	DL3GTGAAAAGGTGTGT
7	2	DL3GGGGCGGGTA	13	5	DL3GGTGTGTCTGTAG
8	2	DL3GCGGGTAGGAT	14	5	DL3TGTGTCTGTAGTA
9	2	DL3GGTAGGATGGGT	15	5	DL3GTAGTATTGTTTTT
10	2	DL3GGATGGGTCTGTG	16	5	DL3AGTATTGTTTTTTT
10	11	DL3GGTCTGTGTGTGT	0	6	DL3CCTCGTGGGATA
12	2	DL3GTGTGTGTGGCG	1	6	DL3TGGGATACAGCG
13	2	DL3TGTGGCGACGAT	2	6	DL3GATACAGCGTCAT
14	2	DL3GACGATTGGGGT	3	6	DL3GCGTCATAGACAG
15	2	DL3ATTGGGGTATGG	4	6	DL3AGACAGAACTAA
15	16	DL3GTATGGGGCTTG	5	6	DL3CAGAACTAAGGA
0	3	DL3GGATTGTGGTCG	6	6	DL3TAAGGACGGAGT
1	3	DL3TGGTCTGGATTGG	7	6	DL3GACGGAGTAGGA
2	3	DL3GGATTGGTCTAAA	8	6	DL3GTAGGATAATAAA
3	3	DL3TCTAAAGTTTAAA	9	6	DL3TAATAAATAGCG
20	4	DL3GTTTAAAAATAGAA	10	6	DL3ATAGCGTAGGAT
5	3	DL3ATAGAAAAACCG	11	6	DL3TAGCGTAGGATG
6	3	DL3AGAAAAACCGC	12	6	DL3AGGATGCAAGTT
7	3	DL3AACC GCCATAC	13	6	DL3ATGCAAGTTATAA
8	3	DL3CCATACGTGAAAA	14	6	DL3GTTATAATGTCCG
25	9	DL3ACGTGAAAATTGT	15	6	DL3ATGTCCGCTTGT
10	3	DL3AATTGTCAGTGGG	16	6	DL3TCCGCTTGTATG
11	3	DL3TGTCAGTGGGGG	0	7	DL3GTGAGTGCCCTC
12	3	DL3TGGGGGGTTGA	1	7	DL3TGCCCTCGAGAG
13	3	DL3GGGTTGATTGTGT	2	7	DL3CCTCGAGAGGTA
30	14	DL3TTGTGTAATAAAA	3	7	DL3AGAGGTACGTAA
15	3	DL3AATAAAAGGGGA	4	7	DL3ACGTAAACCATA
16	3	DL3TAAAGGGGAGG	5	7	DL3ACCATAAAAGCAG
0	4	DL3GTTTTTTTAAAGG	6	7	DL3AAAGCAGACCC
1	4	DL3TTTTTAAAGGTGG	7	7	DL3AGACCCCCCAT
35	2	DL3AGGTGGTTTGG	8	7	DL3CCCCCATACGT
3	4	DL3TTGGGGGGGAG	9	7	DL3CATACGTGCGCT
4	4	DL3GGAGGGGGCG	10	7	DL3GTGCGCTATCAG
5	4	DL3GGGGCGAAGAC	11	7	DL3GCGCTATCAGTA
6	4	DL3GAAGACCGGATG	12	7	DL3TCAGTAACGCTC
40	7	DL3CCGGATGTCTGTG	13	7	DL3GTAACGCTCTGC
8	4	DL3GTCTGAATTTGT	14	7	DL3CTCTGCGACCTC
9	4	DL3CGTGAATTTGTGT	15	7	DL3GACCTCGGCCT
10	4	DL3TTGTGTAGAGACG	16	7	DL3TCGGCCTCGTG
11	4	DL3TAGAGACGGTTT	0	8	DL3GATGAAGTCCAG
45	12	DL3ACGGTTTGGGG	1	8	DL3AGTCCCAGTATTT
13	4	DL3TGGGGTTTTTGT	2	8	DL3GTATTTTCGGATTT
14	4	DL3GGGTTTTTGT	3	8	DL3TCGGATTTATCG
15	4	DL3TTGTTTCTTGGG	4	8	DL3GATTTATCGGGT
16	4	DL3TCTTGGGATTGTG	5	8	DL3ATCGGGTGTGCA
50	0	DL3TGTATGAATGATTT	6	8	DL3TGTGCAAGGGGA
1	5	DL3TGATTTACACAA	7	8	DL3CAAGGGGAATTT
2	5	DL3ACACAATTAATTAA	8	8	DL3GAATTTATTCTGTA
3	5	DL3AATTAATTACGAA	9	8	DL3TCTGTAGTGCTAC
4	5	DL3TACGAACATCCTG	10	8	DL3GTAGTGCTACCT
55	5	DL3ACGAACATCCTGT	11	8	DL3GCTACCTAGTAG
6	5	DL3TCCTGTATTATTA	12	8	DL3CTAGTAGTCCAGA
7	5	DL3GTATTATTATTGTT	13	8	DL3TCCAGATAGTGGG

	14	8	DL3AGATAGTGGGATA	8	12	DL3TGTTTCGTTTCATGT
	15	8	DL3GGGATAAATTGGT	9	12	DL3CGTTTCATGTCGTT
	16	8	DL3TAATTGGTGTAGTG	10	12	DL3GTCGTTAGTTGG
	0	9	DL3TATAGGGCGTGT	11	12	DL3TAGTTGGGAGTT
5	1	9	DL3GGCGTGTCTCA	12	12	DL3GGAGTTGATAGTG
	2	9	DL3GTGTTCTCACGAT	13	12	DL3ATAGTGTGTAGTT
	3	9	DL3TCACGATGAGAGG	14	12	DL3GTGTAGTTGACGT
	4	9	DL3ATGAGAGGAGCG	15	12	DL3TGACGTTGAGGT
	5	9	DL3AGGAGCGAGGC	16	12	DL3CGTTGAGGTTTA
10	6	9	DL3CGAGGCCCGG	5	13	DL3TATAACATGCCAT
	7	9	DL3GCCCCGGTATT	6	13	DL3AACATGCCATGGT
	8	9	DL3CGGGTATTGTGA	7	13	DL3CCATGGTATTTAT
	9	9	DL3GTGAACCCCCAT	8	13	DL3ATTTATGAACCTGG
	10	9	DL3CCCCATCGATTT	9	13	DL3AACTGGTGGACAT
15	11	9	DL3ATCGATTTCACTT	10	13	DL3TGGACATCATGTA
	12	9	DL3TTTCACTTGACAT	11	13	DL3CATGTATTTTGG
	13	9	DL3TTGACATAGAGCT	12	13	DL3TTTTGGGTTAGG
	14	9	DL3TAGAGCTGTAGAC	13	13	DL3GGGTTAGGATGT
	15	9	DL3GTAGACCAAGGA	14	13	DL3GGATGTAGTTTGG
20	16	9	DL3ACCAAGGATGAAG	15	13	DL3TGTAGTTTGGG
	0	10	DL3CGTGTAATGTCAG	16	13	DL3TTTGGGGGAGG
	1	10	DL3TGTCAGTTTAGGG	5	14	DL3GGGTTTCATAACTG
	2	10	DL3TCAGTTTAGGGA	6	14	DL3ATAACTGAGTGGG
	3	10	DL3TAGGGAAGAGCA	7	14	DL3AACTGAGTGGGT
25	4	10	DL3AAGAGCAGGGGT	8	14	DL3GTGGGTAGTTGT
	5	10	DL3CAGGGGTACCTA	9	14	DL3GTAGTTGTTGGC
	6	10	DL3GGTACCTACTGG	10	14	DL3GTTGGCGATACA
	7	10	DL3TACTGGGGGGA	11	14	DL3CGATACATAAAAAG
	8	10	DL3GGGGGAGTCTAT	12	14	DL3TAAAAGCATGTAA
30	9	10	DL3AGTCTATCCCCA	13	14	DL3GCATGTAATGACG
	10	10	DL3ATCCCCAGGGA	14	14	DL3ATGACGGTCCGT
	11	10	DL3CAGGGAACTGGT	15	14	DL3GTCGGTGGTACT
	12	10	DL3ACTGGTGGTAGG	16	14	DL3GGTACTTATAACA
	13	10	DL3CTGGTGGTAGGA	5	15	DL3TCGATTCTAAGAT
35	14	10	DL3GTAGGAGGCACA	6	15	DL3TAAGATTAAATTT
	15	10	DL3GGCACATTTAGT	7	15	DL3AAATTTGAATAAG
	16	10	DL3TTTAGTTATAGGG	8	15	DL3AATAAGAGACAAG
	0	11	DL3AGGTTTACGGTG	9	15	DL3AAGAGACAAGAAA
	1	11	DL3TACGGTGGGGA	10	15	DL3AAGAAAGTACCC
40	2	11	DL3GTGGGGAGTGG	11	15	DL3AAAGTACCCCTT
	3	11	DL3GGGAGTGGGTGA	12	15	DL3CCCCTTCGTCTA
	4	11	DL3GGGTGATCCTATG	13	15	DL3CTTCGTCTAAAC
	5	11	DL3CCTATGGTTGTTT	14	15	DL3CTAAACCCATGG
	6	11	DL3GGTTGTTTGGATG	15	15	DL3AACCCATGGTGG
45	7	11	DL3GTTTGGATGGGT	16	15	DL3TGGTGGGTTTCAT
	8	11	DL3ATGGGTGGGAAT	5	16	DL3TTGGAAAAGGT
	9	11	DL3GGGAATTGTCATG	6	16	DL3AAAAGGTTTCCTG
	10	11	DL3GTCATGTATCATGT	7	16	DL3GGTTCCTGTTTA
	11	11	DL3TCATGTATTTCCG	8	16	DL3CCTGTTTAGTCTC
50	12	11	DL3TATTTCCGGTAAA	9	16	DL3TTAGTCTCTTTTT
	13	11	DL3TTCCGGTAAATGG	10	16	DL3CTTTTTCAGAAAT
	14	11	DL3GTAAATGGCATGT	11	16	DL3AGAAATTGAGGTG
	15	11	DL3GCATGTAATCGTG	12	16	DL3AAATTGAGGTGGT
	16	11	DL3GTAATCGTGTAAT	13	16	DL3GGTGGTAATCGT
55	5	12	DL3GGGAGGGGTAC	14	16	DL3TAATCGTGGGTT
	6	12	DL3GGGTACGAATGT	15	16	DL3GTGGGTTTCGAT
	7	12	DL3ACGAATGTTTCGTT	16	16	DL3GGTTCGATTCT

No probes were present in positions X, Y = 0, 12 to X, Y = 4, 12; X, Y = 0, 13 to X, Y = 4, 13; X, Y = 0, 14 to X, Y = 4, 14; X, Y = 0, 15 to X, Y = 4, 15; X, Y = 0, 16 to X, Y = 4, 16;

The length of each of the probes on the chip was variable to minimize differences in melting temperature and potential for cross-hybridization. Each position in the sequence was represented by at least one probe and most positions were represented by 2 or more probes. As noted above, the amount of overlap between the oligonucleotides varied from probe to probe. Figure 35 shows the human mitochondrial genome; "O_H" is the H strand origin of replication, and arrows indicate the cloned unshaded sequence.

DNA was prepared from hair roots of six human donors (mt1 to mt6) and then amplified by PCR and cloned into M13; the resulting clones were sequenced using chain terminators to verify that the desired specific sequences were present. DNA from the sequenced M13 clones was amplified by PCR, transcribed *in vitro*, and labeled with fluorescein-UTP using T3 RNA polymerase. The 1.3 kb RNA transcripts were fragmented and hybridized to the chip. The results showed that each different individual had DNA that produced a unique hybridization fingerprint on the chip and that the differences in the observed patterns could be correlated with differences in the cloned genomic DNA sequence. The results also demonstrated that very long sequences of a target nucleic acid can be represented comprehensively as a specific set of overlapping oligonucleotides and that arrays of such probe sets can be usefully applied to genetic analysis.

The sample nucleic acid was hybridized to the chip in a solution composed of 6 X SSPE, 0.1% Triton-X 100 for 60 minutes at 15°C. The chip was then scanned by confocal scanning fluorescence microscopy. The individual features on the chip were 588 x 588 microns, but the lower left 5 x 5 square features in the array did not contain probes. To quantitate the data, pixel counts were measured within each synthesis site. Pixels represent 50 x 50 microns. The fluorescence intensity for each feature was scaled to a mean

determined from 27 bright features. After scanning, the chip was stripped and rehybridized; all six samples were hybridized to the same chip. Figure 36 shows the image observed from the mt4 sample on the DNA chip. Figure 37 shows the image

5 observed from the mt5 sample on the DNA chip. Figure 38 shows the predicted difference image between the mt4 and mt5 samples on the DNA chip based on mismatches between the two samples and the reference sequence (see Anderson et al., *supra*). Figure 39 shows the actual difference image observed.

10 The results show that, in almost all cases, mismatched probe/target hybrids resulted in lower fluorescence intensity than perfectly matched hybrids. Nonetheless, some probes detected mutations (or specific sequences) better than others, and in several cases, the differences were within noise
15 levels. Improvements can be realized by increasing the amount of overlap between probes and hence overall probe density and, for duplex DNA targets, using a second set of probes, either on the same or a separate chip, corresponding to the second strand of the target. Figure 40, in sheets 1 and 2, shows a
20 plot of normalized intensities across rows 10 and 11 of the array and a tabulation of the mutations detected.

Figure 41 shows the discrimination between wild-type and mutant hybrids obtained with this chip. The median of the six normalized hybridization scores for each probe was taken. The
25 graph plots the ratio of the median score to the normalized hybridization score versus mean counts. On this graph, a ratio of 1.6 and mean counts above 50 yield no false positives, and while it is clear that detection of some mutants can be improved, excellent discrimination is achieved,
30 considering the small size of the array. Figure 42 illustrates how the identity of the base mismatch may influence the ability to discriminate mutant and wild-type sequences more than the position of the mismatch within an oligonucleotide probe. The mismatch position is expressed as
35 % of probe length from the 3'-end. The base change is indicated on the graph. These results show that the DNA chip increases the capacity of the standard reverse dot blot format by orders of magnitude, extending the power of that approach

many fold and that the methods of the invention are more efficient and easier to automate than gel-based methods of nucleic acid sequence and mutation analysis.

To illustrate further these advantages, a second chip was prepared for analyzing a longer segment from human mitochondrial DNA (mtDNA). The chip "tiles" through 648 nucleotides of a reference sequence comprising human H strand mtDNA from positions 16280 to 356, and allows analysis of each nucleotide in the reference sequence. The probes in the array are 15 nucleotides in length, and each position in the target sequence is represented by a set of 4 probes (A, C, G, T substitutions), which differed from one another at position 7 from the 3'-end. The array consists of 13 blocks of 4 x 50 probes: each block scans through 50 nucleotides of contiguous mtDNA sequence. The blocks are separated by blank rows. The 4 corner columns contain control probes; there are a total of 2600 probes in a 1.28 cm x 1.28 cm square area (feature), and each area is 256 x 197 microns.

Target RNA was prepared as above. The RNA was fragmented and hybridized to the oligonucleotide array in a solution composed of 6X SSPE, 0.1% Triton X-100 for 60 minutes at 18°C. Unhybridized material was washed away with buffer, and the chip was scanned at 25 micron pixel resolution.

Figure 43 provides a 5' to 3' sequence listing of one target corresponding to the probes on the chip. X is a control probe. Positions that differ in the target (i.e., are mismatched with the probe at the designated site) are in bold. Figure 44 shows the fluorescence image produced by scanning the chip when hybridized to this sample. About 95% of the sequence could be read correctly from only one strand of the original duplex target nucleic acid. Although some probes did not provide excellent discrimination and some probes did not appear to hybridize to the target efficiently, excellent results were achieved. The target sequence differed from the probe set at six positions: 4 transitions and 2 insertions. All 4 transitions were detected, and specific probes could readily be incorporated into the array to detect insertions or deletions. Figure 45 illustrates the detection of 4

transitions in the target sequence relative to the wild-type probes on the chip.

A further chip was constructed comprising probes tiling across the entire D-loop region (1.3 kb) of mt DNA sequences from two humans. The probes were tiled in rows of four using the basic tiling strategy. The probes were overlapping 15 mers having an interrogation position 7 nucleotides from the 3' end. The complete group of probes tiled on the reference sequence from the first individual, designated mt1, occupied the upper half of the chip. The lower half of the chip contained a similar arrangement based on a second clone, mt2. The probes were synthesized in a 1.28 x 1.28 cm area, which contained a matrix of 115 x 120 cells. The chip contained a total of 10,488 mtDNA probes.

Six samples of target DNA was extracted from hair roots from six individuals. The 1.3 kb region spanning positions 15935 to 667 of human mtDNA was PCR amplified, cloned in bacteriophage M13 and sequenced by conventional methods. The 1.3 kb region was reamplified from the phage clone using primers, L15935-T3, 5'CTCGGAATTAACCCTCACTAAAGGAAACCTTTTCCAAGGA and H667-T7, 5'TAATACGACTCACTATAGGGAGAGGCTAGGACCAAACCTATT tagged with T3 and T7 RNA polymerase promoter sequences. Labelled RNA was generated by *in vitro* transcription using T3 RNA polymerase and fluoresceinated nucleotides, fragmented, and hybridized to the mtDNA control region resequencing chip at room temperature for 60 min, in 6xSSPE + 0.05% triton X-100. Six washes were carried out at room temperature, using 6xSSPE + 0.005% triton X-100, and the chip was read. Signal intensities varied considerably over the chip, but the large dynamic range of the detection system allowed accurate quantitation of intensities over several orders of magnitude. Even relatively low signal intensities yielded accurate results.

Five different clones (mt1-5) were hybridized, each to a separate chip. The reference sequence was also hybridized for comparative purposes. Mean counts per probe cell were determined, and used by automated basecalling software to read the sequence. The accuracy of sequence read from the chip is

summarized as follows. Combining the data from the five targets analyzed, the chip read a total of 6310 nucleotides. Of these nucleotides in the target sequences, 55 were different from the reference sequence (as judged by conventional sequencing) (41 of these 55 nucleotides were both detected and read correctly from the chip). 6 of 55 nucleotides were detected as being ambiguous but their identity could not be read. 2 of 55 nucleotides were detected as mutations, but their identity was miscalled. 6 of 55 nucleotides were incorrectly called as wildtype. Of the 6255 nucleotides in the target sequence that were identical to the reference sequence, only 36 (0.57%) were miscalled or scored as ambiguous.

A further chip was constructed comprising probes tiling across a reference sequence comprising an entire mitochondrial genome. In this chip, a block tiling strategy was used. Each block was designed to analyze seven nucleotides from a target sequence. Each block consisted of four probe sets, the probe sets each having seven probes. A block was laid down on the chip in seven columns of four probes. The upper probe was the same in each column, this being a probe exactly complementary to a subsequence of the reference sequence. The three other probes in each column were identical to the upper probe except in an interrogation position, which was occupied by a different base in each of the four probes in the column. The interrogation position shifted by one position between successive columns. Thus, except for the seven interrogation positions, one in each of the columns of probes, all probes occupying a block were identical. The array comprised many such blocks, each tiled to successive subsequences of the mitochondrial DNA reference sequence. In all, the chip tiled 15,569 nucleotides of reference sequence with double tiling at 42 positions. 66,276 probes occupied an array of 304 x 315 cells, each cell having an area of 42 x 41 microns.

The chip was hybridized to the same target sequences as described for the D-loop region, except that hybridization was at 15°C for 2 hr. The chip was scanned at 5 micron resolution to give an image with approximately 64 pixels per cell. For

blocks of probes tiling across the D-loop region, a sequence-specific hybridization pattern was obtained. For other blocks, only background hybridization was observed.

These results illustrate that longer sequences can be
5 read using the DNA chips and methods of the invention, as
compared to conventional sequencing methods, where reading
length is limited by the resolution of gel electrophoresis.
Hybridization and signal detection require less than an hour
and can be readily shortened by appropriate choice of buffers,
10 temperatures, probes, and reagents.

III. MODES OF PRACTICING THE INVENTION

A. VLSIPS™ Technology

As noted above, the VLSIPS™ technology is described in a
15 number of patent publications and is preferred for making the
oligonucleotide arrays of the invention. A brief description
of how this technology can be used to make and screen DNA
chips is provided in this Example and the accompanying
Figures. In the VLSIPS™ method, light is shone through a mask
20 to activate functional (for oligonucleotides, typically an
-OH) groups protected with a photoremovable protecting group
on a surface of a solid support. After light activation, a
nucleoside building block, itself protected with a
photoremovable protecting group (at the 5'-OH), is coupled to
25 the activated areas of the support. The process can be
repeated, using different masks or mask orientations and
building blocks, to prepare very dense arrays of many
different oligonucleotide probes. The process is illustrated
in Figure 46; Figure 47 illustrates how the process can be
30 used to prepare "nucleoside combinatorials" or
oligonucleotides synthesized by coupling all four nucleosides
to form dimers, trimers and so forth.

New methods for the combinatorial chemical synthesis of
peptide, polycarbamate, and oligonucleotide arrays have
35 recently been reported (see Fodor et al., 1991, *Science* 251:
767-773; Cho et al., 1993, *Science* 261: 1303-1305; and
Southern et al., 1992, *Genomics* 13: 1008-10017, each of which
is incorporated herein by reference). These arrays, or

biological chips (see Fodor et al., 1993, *Nature* 364: 555-556, incorporated herein by reference), harbor specific chemical compounds at precise locations in a high-density, information rich format, and are a powerful tool for the study of biological recognition processes. A particularly exciting application of the array technology is in the field of DNA sequence analysis. The hybridization pattern of a DNA target to an array of shorter oligonucleotide probes is used to gain primary structure information of the DNA target. This format has important applications in sequencing by hybridization, DNA diagnostics and in elucidating the thermodynamic parameters affecting nucleic acid recognition.

Conventional DNA sequencing technology is a laborious procedure requiring electrophoretic size separation of labeled DNA fragments. An alternative approach, termed Sequencing By Hybridization (SBH), has been proposed (Lysov et al., 1988, *Dokl. Akad. Nauk SSSR* 303:1508-1511; Bains et al., 1988, *J. Theor. Biol.* 135:303-307; and Drmanac et al., 1989, *Genomics* 4:114-128, incorporated herein by reference). This method uses a set of short oligonucleotide probes of defined sequence to search for complementary sequences on a longer target strand of DNA. The hybridization pattern is used to reconstruct the target DNA sequence. It is envisioned that hybridization analysis of large numbers of probes can be used to sequence long stretches of DNA. In immediate applications of this hybridization methodology, a small number of probes can be used to interrogate local DNA sequence.

The strategy of SBH can be illustrated by the following example. A 12-mer target DNA sequence, AGCCTAGCTGAA, is mixed with a complete set of octanucleotide probes. If only perfect complementarity is considered, five of the 65,536 octamer probes -TCGGATCG, CGGATCGA, GGATCGAC, GATCGACT, and ATCGACTT will hybridize to the target. Alignment of the overlapping sequences from the hybridizing probes reconstructs the complement of the original 12-mer target:

```
TCGGATCG
CGGATCGA
GGATCGAC
GATCGACT
```

ATCGACTT
TCGGATCGACTT

Hybridization methodology can be carried out by attaching
5 target DNA to a surface. The target is interrogated with a
set of oligonucleotide probes, one at a time (see Strezoska et
al., 1991, *Proc. Natl. Acad. Sci. USA* 88:10089-10093, and
Drmanac et al., 1993, *Science* 260:1649-1652, each of which is
incorporated herein by reference). This approach can be
10 implemented with well established methods of immobilization
and hybridization detection, but involves a large number of
manipulations. For example, to probe a sequence utilizing a
full set of octanucleotides, tens of thousands of
hybridization reactions must be performed. Alternatively, SBH
15 can be carried out by attaching probes to a surface in an
array format where the identity of the probes at each site is
known. The target DNA is then added to the array of probes.
The hybridization pattern determined in a single experiment
directly reveals the identity of all complementary probes.

20 As noted above, a preferred method of oligonucleotide
probe array synthesis involves the use of light to direct the
synthesis of oligonucleotide probes in high-density,
miniaturized arrays. Photolabile 5'-protected
N-acyl-deoxynucleoside phosphoramidites, surface linker
25 chemistry, and versatile combinatorial synthesis strategies
have been developed for this technology. Matrices of
spatially-defined oligonucleotide probes have been generated,
and the ability to use these arrays to identify complementary
sequences has been demonstrated by hybridizing fluorescent
30 labeled oligonucleotides to the DNA chips produced by the
methods. The hybridization pattern demonstrates a high degree
of base specificity and reveals the sequence of
oligonucleotide targets.

The basic strategy for light-directed oligonucleotide
35 synthesis (1) is outlined in Fig. 46. The surface of a solid
support modified with photolabile protecting groups (X) is
illuminated through a photolithographic mask, yielding
reactive hydroxyl groups in the illuminated regions. A
3'-O-phosphoramidite activated deoxynucleoside (protected at

the 5'-hydroxyl with a photolabile group) is then presented to the surface and coupling occurs at sites that were exposed to light. Following capping, and oxidation, the substrate is rinsed and the surface illuminated through a second mask, to
5 expose additional hydroxyl groups for coupling. A second 5'-protected, 3'-O-phosphoramidite activated deoxynucleoside is presented to the surface. The selective photodeprotection and coupling cycles are repeated until the desired set of products is obtained.

10 Light directed chemical synthesis lends itself to highly efficient synthesis strategies which will generate a maximum number of compounds in a minimum number of chemical steps. For example, the complete set of 4^n polynucleotides (length n), or any subset of this set can be produced in only $4 \times n$
15 chemical steps. See Fig. 47. The patterns of illumination and the order of chemical reactants ultimately define the products and their locations. Because photolithography is used, the process can be miniaturized to generate high-density arrays of oligonucleotide probes. For an example of the
20 nomenclature useful for describing such arrays, an array containing all possible octanucleotides of dA and dT is written as $(A+T)^8$. Expansion of this polynomial reveals the identity of all 256 octanucleotide probes from AAAAAAAAAA to TTTTTTTT. A DNA array composed of complete sets of
25 dinucleotides is referred to as having a complexity of 2. The array given by $(A+T+C+G)^8$ is the full 65,536 octanucleotide array of complexity four. Computer-aided methods of laying down predesigned arrays of probes using VLSIPS™ technology are described in commonly-assigned co-pending application USSN
30 08/249,188, filed May 24, 1994 (incorporated by reference in its entirety for all purposes).

To carry out hybridization of DNA targets to the probe arrays, the arrays are mounted in a thermostatically controlled hybridization chamber. Fluorescein labeled DNA
35 targets are injected into the chamber and hybridization is allowed to proceed for 5 min to 24 hr. The surface of the matrix is scanned in an epifluorescence microscope (Zeiss Axioscop 20) equipped with photon counting electronics using

50 - 100 μ W of 488 nm excitation from an Argon ion laser (Spectra Physics Model 2020). Measurements may be made with the target solution in contact with the probe matrix or after washing. Photon counts are stored and image files are presented after conversion to an eight bit image format. See Fig. 51.

When hybridizing a DNA target to an oligonucleotide array, $N = L_t - (L_p - 1)$ complementary hybrids are expected, where N is the number of hybrids, L_t is the length of the DNA target, and L_p is the length of the oligonucleotide probes on the array. For example, for an 11-mer target hybridized to an octanucleotide array, $N = 4$. Hybridizations with mismatches at positions that are 2 to 3 residues from either end of the probes will generate detectable signals. Modifying the above expression for N , one arrives at a relationship estimating the number of detectable hybridizations (N_d) for a DNA target of length L_t and an array of complexity C . Assuming an average of 5 positions giving signals above background:

$$N_d = (1 + 5(C-1))[L_t - (L_p - 1)].$$

Arrays of oligonucleotides can be efficiently generated by light-directed synthesis and can be used to determine the identity of DNA target sequences. Because combinatorial strategies are used, the number of compounds increases exponentially while the number of chemical coupling cycles increases only linearly. For example, synthesizing the complete set of 4^8 (65,536) octanucleotides will add only four hours to the synthesis for the 16 additional cycles. Furthermore, combinatorial synthesis strategies can be implemented to generate arrays of any desired composition. For example, because the entire set of dodecamers (4^{12}) can be produced in 48 photolysis and coupling cycles (b^n compounds requires $b \times n$ cycles), any subset of the dodecamers (including any subset of shorter oligonucleotides) can be constructed with the correct lithographic mask design in 48 or fewer chemical coupling steps. In addition, the number of compounds in an array is limited only by the density of synthesis sites and the overall array size. Recent experiments have demonstrated hybridization to probes

synthesized in 25 μm sites. At this resolution, the entire set of 65,536 octanucleotides can be placed in an array measuring 0.64 cm square, and the set of 1,048,576 dodecanucleotides requires only a 2.56 cm array.

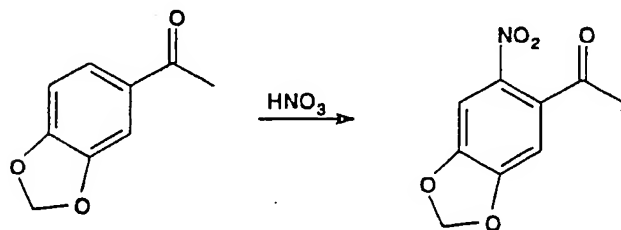
5 Genome sequencing projects will ultimately be limited by DNA sequencing technologies. Current sequencing methodologies are highly reliant on complex procedures and require substantial manual effort. Sequencing by hybridization has the potential for transforming many of the manual efforts into
10 more efficient and automated formats. Light-directed synthesis is an efficient means for large scale production of miniaturized arrays for SBH. The oligonucleotide arrays are not limited to primary sequencing applications. Because single base changes cause multiple changes in the
15 hybridization pattern, the oligonucleotide arrays provide a powerful means to check the accuracy of previously elucidated DNA sequence, or to scan for changes within a sequence. In the case of octanucleotides, a single base change in the target DNA results in the loss of eight complements, and
20 generates eight new complements. Matching of hybridization patterns may be useful in resolving sequencing ambiguities from standard gel techniques, or for rapidly detecting DNA mutational events. The potentially very high information content of light-directed oligonucleotide arrays will change
25 genetic diagnostic testing. Sequence comparisons of hundreds to thousands of different genes will be assayed simultaneously instead of the current one, or few at a time format. Custom arrays can also be constructed to contain genetic markers for the rapid identification of a wide variety of pathogenic
30 organisms.

Oligonucleotide arrays can also be applied to study the sequence specificity of RNA or protein-DNA interactions. Experiments can be designed to elucidate specificity rules of non Watson-Crick oligonucleotide structures or to investigate
35 the use of novel synthetic nucleoside analogs for antisense or triple helix applications. Suitably protected RNA monomers may be employed for RNA synthesis. The oligonucleotide arrays should find broad application deducing the thermodynamic and

kinetic rules governing formation and stability of oligonucleotide complexes.

Other than the use of photoremovable protecting groups, the nucleoside coupling chemistry is very similar to that used routinely today for oligonucleotide synthesis. Fig. 48 shows the deprotection, coupling, and oxidation steps of a solid phase DNA synthesis method. Fig. 49 shows an illustrative synthesis route for the nucleoside building blocks used in the method. Fig. 50 shows a preferred photoremovable protecting group, MeNPOC, and how to prepare the group in active form. The procedures described below show how to prepare these reagents. The nucleoside building blocks are 5'-MeNPOC-THYMIDINE-3'-OCEP; 5'-MeNPOC-N⁴-t-BUTYL PHENOXYACETYL-DEOXYCYTIDINE-3'-OCEP; 5'-MeNPOC-N⁴-t-BUTYL PHENOXYACETYL-DEOXYGUANOSINE-3'-OCEP; and 5'-MeNPOC-N⁴-t-BUTYL PHENOXYACETYL-DEOXYADENOSINE-3'-OCEP..

1. Preparation of 4,5-methylenedioxy-2-nitroacetophenone

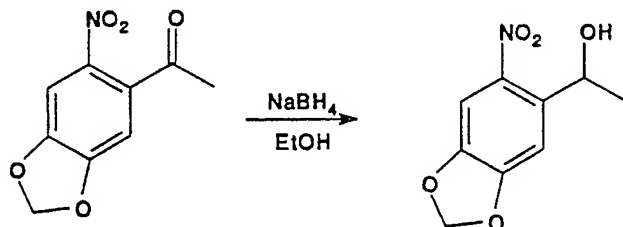


20

A solution of 50 g (0.305 mole) 3,4-methylenedioxyacetophenone (Aldrich) in 200 mL glacial acetic acid was added dropwise over 30 minutes to 700 mL of cold (2-4°C) 70% HNO₃ with stirring (NOTE: the reaction will overheat without external cooling from an ice bath, which can be dangerous and lead to side products). At temperatures below 0°C, however, the reaction can be sluggish. A temperature of 3-5°C seems to be optimal). The mixture was left stirring for another 60 minutes at 3-5°C, and then allowed to approach ambient temperature. Analysis by TLC (25% EtOAc in hexane) indicated complete conversion of the starting material within 1-2 hr. When the reaction was complete, the mixture was poured into ~3 liters of crushed ice, and the resulting yellow solid was

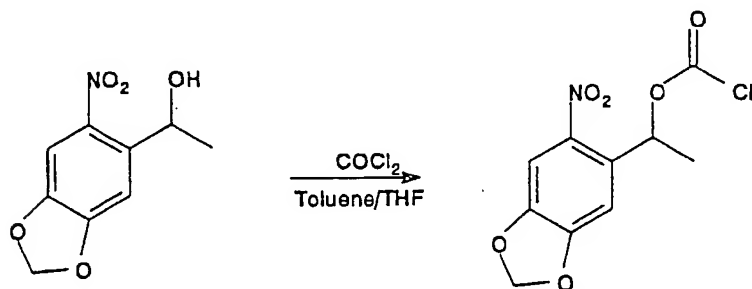
filtered off, washed with water and then suction-dried. Yield ~53 g (84%), used without further purification.

2. Preparation of 1-(4,5-Methylenedioxy-2-nitrophenyl)
5 ethanol



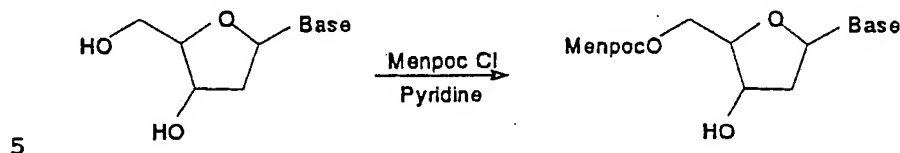
Sodium borohydride (10g; 0.27 mol) was added slowly to a cold,
10 stirring suspension of 53g (0.25 mol) of
4,5-methylenedioxy-2-nitroacetophenone in 400 mL methanol.
The temperature was kept below 10°C by slow addition of the
NaBH₄ and external cooling with an ice bath. Stirring was
continued at ambient temperature for another two hours, at
15 which time TLC (CH₂Cl₂) indicated complete conversion of the
ketone. The mixture was poured into one liter of ice-water
and the resulting suspension was neutralized with ammonium
chloride and then extracted three times with 400 mL CH₂Cl₂ or
EtOAc (the product can be collected by filtration and washed
20 at this point, but it is somewhat soluble in water and this
results in a yield of only ~60%). The combined organic
extracts were washed with brine, then dried with MgSO₄ and
evaporated. The crude product was purified from the main
byproduct by dissolving it in a minimum volume of CH₂Cl₂ or
25 THF (~175 ml) and then precipitating it by slowly adding hexane
(1000 ml) while stirring (yield 51g; 80% overall). It can
also be recrystallized (e.g., toluene-hexane), but this
reduces the yield.

3. Preparation of 1-(4,5-methylenedioxy-2-nitrophenyl) ethyl chloroformate (MenPOC-Cl)



- 5 Phosgene (500 mL of 20% w/v in toluene from Fluka: 965 mmole; 4 eq.) was added slowly to a cold, stirring solution of 50g (237 mmole; 1 eq.) of 1-(4,5-methylenedioxy-2-nitrophenyl) ethanol in 400 mL dry THF. The solution was stirred overnight
- 10 at ambient temperature at which point TLC (20% Et₂O/hexane) indicated >95% conversion. The mixture was evaporated (an oil-less pump with downstream aqueous NaOH trap is recommended to remove the excess phosgene) to afford a viscous brown oil. Purification was effected by flash chromatography on a short
- 15 (9 x 13 cm) column of silica gel eluted with 20% Et₂O/hexane. Typically 55g (85%) of the solid yellow MenPOC-Cl is obtained by this procedure. The crude material has also been recrystallized in 2-3 crops from 1:1 ether/hexane. On this scale, ~100ml is used for the first crop, with a few percent
- 20 THF added to aid dissolution, and then cooling overnight at -20°C (this procedure has not been optimized). The product should be stored desiccated at -20°C.

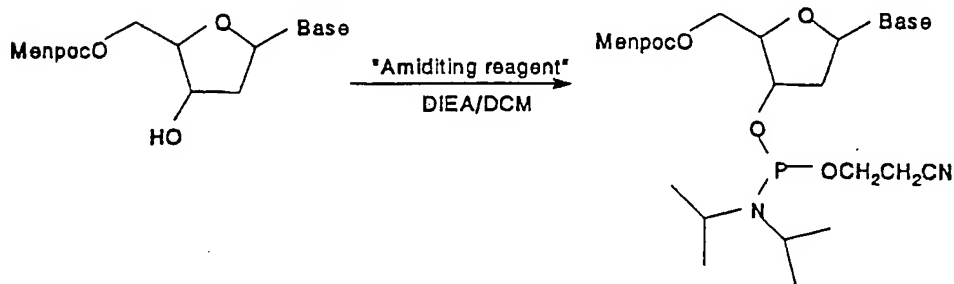
4. Synthesis of 5'-Menpoc-2'-deoxynucleoside-3'-(N,N-diisopropyl 2-cyanoethyl phosphoramidites
(a.) 5'-MeNPOC-Nucleosides



Base= THYMIDINE (T); N-4-ISOBUTYRYL 2'-DEOXYCYTIDINE (ibu-dC);
 N-2-PHENOXYACETYL 2'DEOXYGUANOSINE (PAC-dG); and
 10 N-6-PHENOXYACETYL 2'DEOXYADENOSINE (PAC-dA)

All four of the 5'-MenPOC nucleosides were prepared from the base-protected 2'-deoxynucleosides by the following procedure. The protected 2'-deoxynucleoside (90 mmole) was dried by
 15 co-evaporating twice with 250 mL anhydrous pyridine. The nucleoside was then dissolved in 300 mL anhydrous pyridine (or 1:1 pyridine/DMF, for the dG^{PAC} nucleoside) under argon and cooled to -2°C in an ice bath. A solution of 24.6g (90 mmole) MenPOC-Cl in 100 mL dry THF was then added with
 20 stirring over 30 minutes. The ice bath was removed, and the solution allowed to stir overnight at room temperature (TLC: 5-10% MeOH in CH₂Cl₂; two diastereomers). After evaporating the solvents under vacuum, the crude material was taken up in 250 mL ethyl acetate and extracted with saturated aqueous
 25 NaHCO₃ and brine. The organic phase was then dried over Na₂SO₄, filtered and evaporated to obtain a yellow foam. The crude products were finally purified by flash chromatography (9 x 30 cm silica gel column eluted with a stepped gradient of 2% - 6% MeOH in CH₂Cl₂). Yields of the purified diastereomeric
 30 mixtures are in the range of 65-75%.

(b.) 5'- Menpoc-2'-deoxynucleoside-3'-(N,N-diisopropyl 2-cyanoethyl phosphoramidites)



5

The four deoxynucleosides were phosphitylated using either 2-cyanoethyl- N,N- diisopropyl chlorophosphoramidite, or 2-cyanoethyl- N,N,N',N'- tetraisopropylphosphorodiamidite. The following is a typical procedure. Add 16.6g (17.4 ml; 55 mmole) of 2- cyanoethyl- N,N,N',N'- tetraisopropylphosphorodiamidite to a solution of 50 mmole 5'- MenPOC-nucleoside and 4.3g (25 mmole) diisopropylammonium tetrazolide in 250 mL dry CH₂Cl₂ under argon at ambient temperature. Continue stirring for 4-16 hours (reaction monitored by TLC: 45:45:10 hexane/CH₂Cl₂/Et₃N). Wash the organic phase with saturated aqueous NaHCO₃ and brine, then dry over Na₂SO₄, and evaporate to dryness. Purify the crude amidite by flash chromatography (9 x 25 cm silica gel column eluted with hexane/CH₂Cl₂/TEA - 45:45:10 for A, C, T; or 0:90:10 for G). The yield of purified amidite is about 90%.

B. PREPARATION OF LABELED DNA/HYBRIDIZATION TO ARRAY

25

1. PCR

PCR amplification reactions are typically conducted in a mixture composed of, per reaction: 1 μl genomic DNA; 10 μl each primer (10 pmol/μl stocks); 10 μl 10 x PCR buffer (100 mM Tris.Cl pH8.5, 500 mM KCl, 15 mM MgCl₂); 10 μl 2 mM dNTPs (made from 100 mM dNTP stocks); 2.5 U Taq polymerase (Perkin Elmer AmpliTaq™, 5 U/μl); and H₂O to 100 μl. The cycling conditions are usually 40 cycles (94°C 45 sec, 55°C 30 sec, 72°C 60 sec) but may need to be varied considerably from

30

sample type to sample type. These conditions are for 0.2 mL thin wall tubes in a Perkin Elmer 9600 thermocycler. See Perkin Elmer 1992/93 catalogue for 9600 cycle time information. Target, primer length and sequence composition, among other factors, may also affect parameters.

For products in the 200 to 1000 bp size range, check 2 μ l of the reaction on a 1.5% 0.5x TBE agarose gel using an appropriate size standard (ϕ X174 cut with *Hae*III is convenient). The PCR reaction should yield several picomoles of product. It is helpful to include a negative control (i.e., 1 μ l TE instead of genomic DNA) to check for possible contamination. To avoid contamination, keep PCR products from previous experiments away from later reactions, using filter tips as appropriate. Using a set of working solutions and storing master solutions separately is helpful, so long as one does not contaminate the master stock solutions.

For simple amplifications of short fragments from genomic DNA it is, in general, unnecessary to optimize Mg^{2+} concentrations. A good procedure is the following: make a master mix minus enzyme; dispense the genomic DNA samples to individual tubes or reaction wells; add enzyme to the master mix; and mix and dispense the master solution to each well, using a new filter tip each time.

2. PURIFICATION

Removal of unincorporated nucleotides and primers from PCR samples can be accomplished using the Promega Magic PCR Preps DNA purification kit. One can purify the whole sample, following the instructions supplied with the kit (proceed from section IIIB, 'Sample preparation for direct purification from PCR reactions'). After elution of the PCR product in 50 μ l of TE or H_2O , one centrifuges the eluate for 20 sec at 12,000 rpm in a microfuge and carefully transfers 45 μ l to a new microfuge tube, avoiding any visible pellet. Resin is sometimes carried over during the elution step. This transfer prevents accidental contamination of the linear amplification reaction with 'Magic PCR' resin. Other methods, e.g., size exclusion chromatography, may also be used.

3. Linear amplification

In a 0.2 mL thin-wall PCR tube mix: 4 μ l purified PCR product; 2 μ l primer (10 pmol/ μ l); 4 μ l 10 x PCR buffer; 4 μ l dNTPs (2 mM dA, dC, dG, 0.1 mM dT); 4 μ l 0.1 mM dUTP; 1 μ l 1 mM fluorescein dUTP (Amersham RPN 2121); 1 U Taq polymerase (Perkin Elmer, 5 U/ μ l); and add H₂O to 40 μ l. Conduct 40 cycles (92°C 30 sec, 55°C 30 sec, 72°C 90 sec) of PCR. These conditions have been used to amplify a 300 nucleotide mitochondrial DNA fragment but are applicable to other fragments. Even in the absence of a visible product band on an agarose gel, there should still be enough product to give an easily detectable hybridization signal. If one is not treating the DNA with uracil DNA glycosylase (see Section 4), dUTP can be omitted from the reaction.

15

4. Fragmentation

Purify the linear amplification product using the Promega Magic PCR Preps DNA purification kit, as per Section 2 above. In a 0.2 mL thin-wall PCR tube mix: 40 μ l purified labeled DNA; 4 μ l 10 x PCR buffer; and 0.5 μ l uracil DNA glycosylase (BRL 1U/ μ l). Incubate the mixture 15 min at 37°C, then 10 min at 97°C; store at -20°C until ready to use.

5. Hybridization, Scanning & Stripping

A blank scan of the slide in hybridization buffer only is helpful to check that the slide is ready for use. The buffer is removed from the flow cell and replaced with 1 mL of (fragmented) DNA in hybridization buffer and mixed well. The scan is performed in the presence of the labeled target. Fig. 51 illustrates an illustrative detection system for scanning a DNA chip. A series of scans at 30 min intervals using a hybridization temperature of 25°C yields a very clear signal, usually in at least 30 min to two hours, but it may be desirable to hybridize longer, i.e., overnight. Using a laser power of 50 μ W and 50 μ m pixels, one should obtain maximum counts in the range of hundreds to low thousands/pixel for a new slide. When finished, the slide can be stripped using 50%

formamide. rinsing well in deionized H₂O, blowing dry, and storing at room temperature.

C. PREPARATION OF LABELED RNA/HYBRIDIZATION TO ARRAY

5 1. Tagged primers

The primers used to amplify the target nucleic acid should have promoter sequences if one desires to produce RNA from the amplified nucleic acid. Suitable promoter sequences are shown below and include:

10 (1) the T3 promoter sequence:

5'-CGGAATTAACCCTCACTAAAGG

5'-AATTAACCCTCACTAAAGGGAG;

(2) the T7 promoter sequence:

5' TAATACGACTCACTATAGGGAG;

15 and (3) the SP6 promoter sequence:

5' ATTTAGGTGACACTATAGAA.

The desired promoter sequence is added to the 5' end of the PCR primer. It is convenient to add a different promoter to
20 each primer of a PCR primer pair so that either strand may be transcribed from a single PCR product.

Synthesize PCR primers so as to leave the DMT group on. DMT-on purification is unnecessary for PCR but appears to be important for transcription. Add 25 μ l 0.5M NaOH to
25 collection vial prior to collection of oligonucleotide to keep the DMT group on. Deprotect using standard chemistry -- 55°C overnight is convenient.

HPLC purification is accomplished by drying down the oligonucleotides, resuspending in 1 mL 0.1 M TEAA (dilute 2.0
30 M stock in deionized water, filter through 0.2 micron filter) and filter through 0.2 micron filter. Load 0.5 mL on reverse phase HPLC (column can be a Hamilton PRP-1 semi-prep, #79426). The gradient is 0 -> 50% CH₃CN over 25 min (program 0.2 μ mol.prep.0-50, 25 min). Pool the desired fractions, dry down,
35 resuspend in 200 μ l 80% HAC. 30 min RT. Add 200 μ l EtOH; dry down. Resuspend in 200 μ l H₂O, plus 20 μ l NaAc pH5.5, 600 μ l EtOH. Leave 10 min on ice; centrifuge 12,000 rpm for 10 min in microfuge. Pour off supernatant. Rinse pellet with 1 mL

EtOH, dry, resuspend in 200 μ l H₂O. Dry, resuspend in 200 μ l TE. Measure A₂₆₀, prepare a 10 pmol/ μ l solution in TE (10 mM Tris.Cl pH 8.0, 0.1 mM EDTA). Following HPLC purification of a 42 mer, a yield in the vicinity of 15 nmol from a 0.2 μ mol scale synthesis is typical.

2. Genomic DNA Preparation

Add 500 μ l (10 mM Tris.Cl pH8.0, 10 mM EDTA, 100 mM NaCl, 2% (w/v) SDS, 40 mM DTT, filter sterilized) to the sample. Add 1.25 μ l 20 mg/ml proteinase K (Boehringer) Incubate at 55°C for 2 hours, vortexing once or twice. Perform 2x 0.5 mL 1:1 phenol:CHCl₃ extractions. After each extraction, centrifuge 12,000 rpm 5 min in a microfuge and recover 0.4 mL supernatant. Add 35 μ l NaAc pH5.2 plus 1 mL EtOH. Place sample on ice 45 min; then centrifuge 12,000 rpm 30 min, rinse, air dry 30 min, and resuspend in 100 μ l TE.

3. PCR

PCR is performed in a mixture containing, per reaction: 1 μ l genomic DNA; 4 μ l each primer (10 pmol/ μ l stocks); 4 μ l 10 x PCR buffer (100 mM Tris.Cl pH8.5, 500 mM KCl, 15 mM MgCl₂); 4 μ l 2 mM dNTPs (made from 100 mM dNTP stocks); 1 U Taq polymerase (Perkin Elmer, 5 U/ μ l); H₂O to 40 μ l. About 40 cycles (94°C 30 sec, 55°C 30 sec, 72°C 30 sec) are performed, but cycling conditions may need to be varied. These conditions are for 0.2 mL thin wall tubes in Perkin Elmer 9600. For products in the 200 to 1000 bp size range, check 2 μ l of the reaction on a 1.5% 0.5xTBE agarose gel using an appropriate size standard. For larger or smaller volumes (20 - 100 μ l), one can use the same amount of genomic DNA but adjust the other ingredients accordingly.

4. In vitro transcription

Mix: 3 μ l PCR product; 4 μ l 5x buffer; 2 μ l DTT; 2.4 μ l 10 mM rNTPs (100 mM solutions from Pharmacia); 0.48 μ l 10 mM fluorescein-UTP (Fluorescein-12-UTP, 10 mM solution, from Boehringer Mannheim); 0.5 μ l RNA polymerase (Promega T3 or T7 RNA polymerase); and add H₂O to 20 μ l. Incubate at 37°C for 3

h. Check 2 μ l of the reaction on a 1.5% 0.5xTBE agarose gel using a size standard. 5x buffer is 200 mM Tris pH 7.5, 30 mM $MgCl_2$, 10 mM spermidine, 50 mM NaCl, and 100 mM DTT (supplied with enzyme). The PCR product needs no purification and can be added directly to the transcription mixture. A 20 μ l reaction is suggested for an initial test experiment and hybridization; a 100 μ l reaction is considered "preparative" scale (the reaction can be scaled up to obtain more target). The amount of PCR product to add is variable; typically a PCR reaction will yield several picomoles of DNA. If the PCR reaction does not produce that much target, then one should increase the amount of DNA added to the transcription reaction (as well as optimize the PCR). The ratio of fluorescein-UTP to UTP suggested above is 1:5, but ratios from 1:3 to 1:10 - all work well. One can also label with biotin-UTP and detect with streptavidin-FITC to obtain similar results as with fluorescein-UTP detection.

For nondenaturing agarose gel electrophoresis of RNA, note that the RNA band will normally migrate somewhat faster than the DNA template band, although sometimes the two bands will comigrate. The temperature of the gel can effect the migration of the RNA band. The RNA produced from *in vitro* transcription is quite stable and can be stored for months (at least) at $-20^{\circ}C$ without any evidence of degradation. It can be stored in unsterilized 6xSSPE 0.1% triton X-100 at $-20^{\circ}C$ for days (at least) and reused twice (at least) for hybridization, without taking any special precautions in preparation or during use. RNase contamination should of course be avoided. When extracting RNA from cells, it is preferable to work very rapidly and to use strongly denaturing conditions. Avoid using glassware previously contaminated with RNases. Use of new disposable plasticware (not necessarily sterilized) is preferred, as new plastic tubes, tips, etc., are essentially RNase free. Treatment with DEPC or autoclaving is typically not necessary.

5. Fragmentation

Heat transcription mixture at 94 degrees for forty min. The extent of fragmentation is controlled by varying Mg^{2+} concentration (30 mM is typical), temperature, and duration of heating.

6. Hybridization, Scanning, & Stripping

A blank scan of the slide in hybridization buffer only is helpful to check that the slide is ready for use. The buffer is removed from the flow cell and replaced with 1 mL of (hydrolysed) RNA in hybridization buffer and mixed well. Incubate for 15 - 30 min at 18°C. Remove the hybridization solution, which can be saved for subsequent experiments. Rinse the flow cell 4 - 5 times with fresh changes of 6 x SSPE / 0.1% Triton X-100, equilibrated to 18°C. The rinses can be performed rapidly, but it is important to empty the flow cell before each new rinse and to mix the liquid in the cell thoroughly. A series of scans at 30 min intervals using a hybridization temperature of 25°C yields a very clear signal, usually in at least 30 min to two hours, but it may be desirable to hybridize longer, i.e., overnight. Using a laser power of 50 μ W and 50 μ m pixels, one should obtain maximum counts in the range of hundreds to low thousands/pixel for a new slide. When finished, the slide can be stripped using warm water.

These conditions are illustrative and assume a probe length of ~15 nucleotides. The stripping conditions suggested are fairly severe, but some signal may remain on the slide if the washing is not stringent. Nevertheless, the counts remaining after the wash should be very low in comparison to the signal in presence of target RNA. In some cases, much gentler stripping conditions are effective. The lower the hybridization temperature and the longer the duration of hybridization, the more difficult it is to strip the slide. Longer targets may be more difficult to strip than shorter targets.

7. Amplification of Signal

A variety of methods can be used to enhance detection of labelled targets bound to a probe on the array. In one

embodiment, the protein MutS (from E. coli) or equivalent proteins such as yeast MSH1, MSH2, and MSH3; mouse Rep-3, and Streptococcus Hex-A, is used in conjunction with target hybridization to detect probe-target complex that contain mismatched base pairs. The protein, labeled directly or indirectly, can be added to the chip during or after hybridization of target nucleic acid, and differentially binds to homo- and heteroduplex nucleic acid. A wide variety of dyes and other labels can be used for similar purposes. For instance, the dye YOYO-1 is known to bind preferentially to nucleic acids containing sequences comprising runs of 3 or more G residues.

8. Detection of Repeat Sequences

In some circumstances, i.e., target nucleic acids with repeated sequences or with high G/C content, very long probes are sometimes required for optimal detection. In one embodiment for detecting specific sequences in a target nucleic acid with a DNA chip, repeat sequences are detected as follows. The chip comprises probes of length sufficient to extend into the repeat region varying distances from each end. The sample, prior to hybridization, is treated with a labelled oligonucleotide that is complementary to a repeat region but shorter than the full length of the repeat. The target nucleic is labelled with a second, distinct label. After hybridization, the chip is scanned for probes that have bound both the labelled target and the labelled oligonucleotide probe; the presence of such bound probes shows that at least two repeat sequences are present.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. All publications and patent documents cited in this application are incorporated by reference in their entirety for all

purposes to the same extent as if each individual publication or patent document were so individually denoted.

Mutation	Exon	Ex Size	Pop Freq	Location	Sequence Around Mutation Site	PRIMERS	Amp Sz
297-3 C>T	12	109		Sub C>T +3 Exon 3	CTTTTATTCTTTTG(C>T)AGAGAAATGGGATAGA	787/788	297
R750	3	109	Manchester	Substitute G>A at 60	TAATGCOCTTGGGC(G>A)ATGTTTTTCTGGGA	787/788	297
300 del A	3	109	Manchester	Delete A at 4	ATTCTTTTGCAGAGAAATGGGATAGAGAGCTGGCT	787/788	297
E90X	3	109	Manchester	Substitute G>T at 14	GAATGGGATAGAG(G>T)AGCTGGCTTCAAGA	787/788	297
L88S	3	109	Manchester	Substitute T>C at 99	CTATGGAATCTTTT(T>C)ATATTAGGGGTAAAG	787/788	297
G95E	3	109	0.70%	Substitute G>A at 90	TTATGTTCTATG(G>A)ATCTTTTATATTTAG	787/788	297
R117H	4	216	0.80%	Substitute G>A at 77	AACAAGGAGGAAC(G>A)CTCTATCGGATTTAT	851/769	381
R117C	4	216	rare	Substitute C>T at 76	AACAAGGAGGAAC(C>T)CTCTATCGGATTTAT	851/769	381
Y122X	4	216	0.30%	Substitute T>A at 93	TATCGGATTTA(T>A)CTAGGCATAGGCTTATG	851/769	381
I148T	4	216	Fr Can (10%)	Substitute T>C at 170	GGCCTTCATCACA(T>C)TGGAAATGACAGTGAAGA	851/769	381
621+1G>T	14	216	1.30%	Sub G>T after last base	GATTTATAAGAA(G>T)TAATAGTCTGCTTGCAC	851/769	381
711+1G>T	15	90	0.90%	Sub G>T after last base	CAAAATTTGATGA(G>T)TATGACCTATTGATT	887/888	289
L206W	6a	164	Fr Can (10%)	Substitute T>G at 38	TGGATCGCTCCTT(T>G)CAAGTGGCACTGCTC	934/935	331
1138 ins G	7	247	Manchester	Insert G at 137	AATCATCTCTCGGAAAGATATTGACCACCATCT	789/790	404
1154 ins TC	7	247	Manchester	Insert TC at 153	TATTCAACCACTCTC(A>T)CTGCTGCTTGT	789/790	404
1161 del C	7	247	Manchester	Delete C at 160	CCACCATCTCATTTCTG(A>T)GTTTCTGGCATGG	789/790	404
R334W	7	247	0.40%	Substitute C>T at 131	AAGGAATCATCTCT(C>T)GGAAATATTCTATTA	789/790	404
R347H	7	247	0.10%	Substitute G>A at 171	CTGCATTGTTCTGG(G>A)CATGGCGGTCACTGG	789/790	404
R347L	7	247	rare	Substitute G>T at 171	CTGCATTGTTCTGG(G>T)CATGGCGGTCACTGG	789/790	404
R347P	7	247	0.50%	Substitute G>C at 171	CTGCATTGTTCTGG(G>C)CATGGCGGTCACTGG	789/790	404
1078 del T	7	247	1.10%	Delete T at 77	CTTCTCTCAGGGTCTTCTGGTGTGTTTATC	789/790	404
1248+1 G>A	17	247	Manchester	Sub G>A 1 after Exon 7	AAACAAATACAG(G>A)TAATGTACCATATG	789/790	404
A455E	9	183	0.40%	Substitute C>A at 155	AGGACAGTTGTTGG(C>A)GGTTGCTGATGCA	891/892	386
G480C	10	192	rare	Substitute G>T at 46	GGAGCCTTCAGAG(G>T)GTAAATTAAGCACA	780/850	304
O483X	10	192	0.30%	Substitute C>T at 85	TCATTCTGTTCT(C>T)AGTTTCTCTGGATTAT	780/850	304
D1507	10	192	0.50%	Delete 126, 127, 128	ATTAAGAAATATCCTTTTGGTGTGTTTCTATG	780/850	304
F508C	10	192	rare	Substitute T>G at 131	TAAAGAAATATCATCT(T>G)TGGTGTGTTCTGA	780/850	304
DF508	10	192	67.20%	Delete 129, 130, 131	ATTAAGAAATATCATCTTGGTGTGTTTCTATG	780/850	304
V520F	10	192	0.20%	Substitute G>T at 168	TAGATACAGAAAG(G>T)TCATCAAGCATGCC	780/850	304
1717-1G>A	110	95	1.10%	Sub G>A at-1 Ex11	TATTTTGGTAATA(G>A)GACATCTCCAAGTTT	782/783	233
G542X	11	95	3.40%	Substitute G>T at 40	ACAATATAGTTCTT(C>T)GAGAAAGTGGAAAT	782/783	233
S549N	11	95	rare	Substitute G>A at 62	AGGTGGAATCAGACTGA(G>A)TGGAGGTCAACG	782/783	233
S549I	11	95	rare	Substitute G>T at 62	AGGTGGAATCAGACTGA(G>T)TGGAGGTCAACG	782/783	233
S549R(A>C)	11	95	rare	Substitute A>C at 61	AGGTGGAATCAGACTGA(A>C)TGGAGGTCAACG	782/783	233
S549R(T>G)	11	95	0.30%	Substitute T>G at 63	AGGTGGAATCAGACTGA(T>G)TGGAGGTCAACG	782/783	233
G551D	11	95	2.40%	Substitute G>A at 68	ATCAGACTGAGTGGAG(G>A)TCAAGGACGAAGA	782/783	233
G551S	11	95	rare	Substitute G>A at 67	ATCAGACTGAGTGGAG(G>A)TCAAGGACGAAGA	782/783	233
O552X	11	95	rare	Substitute C>T at 70	ACACTGAGTGGAGGT(C>T)AACGAGCAAGAAAT	782/783	233
R553Q	11	95	rare	Substitute G>A at 74	TGAGTGGAGGTCAAC(G>A)AGCAAGAAATTTCT	782/783	233
R553X	11	95	1.30%	Substitute C>T at 73	TGAGTGGAGGTCAAC(C>T)AGCAAGAAATTTCTT	782/783	233
A559T	11	95	rare	Substitute G>A at 91	GCAAGAAATTTCTTTA(G>A)CAAGGTGAATAAG	782/783	233
R560T	11	95	0.40%	Substitute G>C at 95	AATTTCTTTAGCAAG(G>C)GTGAATAAGTAA	782/783	233
R560K	11	95	rare	Substitute G>A at 95	GAATTTCTTTAGCAAG(G>A)GTGAATAAGTAA	782/783	233
1898+1G>A	112	95	0.90%	Sub G>A after last Ex12	GAAATATTTGAAAG(G>A)TATGTTCTTTGAAAT	931/932	299
D648V	13	724	Nat Am (83%)	Substitute A>T at 177	AAGTCATGGGATGTG(A>T)TTCTTTGACCAAT	955/884	360
2184 del A	13	724	0.70%	Delete A at 286	GACAGAAACAAAAAACAATCTTTTAAACAGAC	955/884	360
2184 ins A	13	724	rare	Insert A after 286	GACAGAAACAAAAAACAATCTTTTAAACAGAC	955/884	360
2789+5G>A	114b	38	1.10%	Sub G>A 5 one after last	CTCCTTGGAAAGTGA(G>A)TATTCATGTGCTA	886/886	374
3272-28A>G	117a	228	rare	Sub A>G 26 before 17b	TTATGTTATTTGCA(A>G)TGTGTTTCTATGGAAA	782/901	414
3272-93T>C	117a	228	rare	Sub T>C 93 before 17b	ATTGTGATATGATTA(T>C)TCTAATTTAGTCTTT	782/901	414
R1086C	17b	228	rare	Substitute C>T at 57	AGGACTATGGAGACTT(C>T)TGTCCTTGGAGCGGC	782/901	414
L1077P	17b	228	rare	Substitute T>C at 91	TTACTTTGAAACT(T>C)GTTCCACAAAGCTC	782/901	414
Y1092X	17b	228	0.50%	Substitute C>A at 137	CCAACTGGTCTTGTGA(C>A)CTGTCAACACTGGG	782/901	414
M1101K	17b	228	Hut (85%)	Substitute T>A at 183	TGGGCTGGTTCCAA(T>A)GAGAAATAGAAATGAT	782/901	414
R1182X	19	249	0.90%	Substitute C>T at 16	ATGCGATCTGTBAG(C>T)GAGTCTTTAAGTTC	784/785	356
3659 del C	19	249	0.80%	Delete C at 59	AAGGTAAACCTACCAAGTCAACCAACCATACA	784/785	356
3849+4 A>G	119	249	1.00%	Sub A>G 4 after last base	TCTGCGCAGAGGGTGA(A>G)GATTTGAACACT	784/785	356
3849+10kb	119	10kb	1.40%	Sub C>T EcoRI Fragment	ATAAAATGG(C>T)GAGTAAGACA	792/791	450
W1282R	20	156	rare	Substitute T>C at 127	AATAACTTTGCAACAG(T>C)GGAGGAAAGCCTTT	784/788	351
W1282S	20	156	2.10%	Substitute G>A at 129	AATAACTTTGCAACAGT(G>A)AGGAAAGCCTTT	784/788	351
3905insT	20	156	2.10%	Insert T at 56	CTTTGTTATCAGCTTTTGTGAGACTACTGAACAC	784/788	351
4005+1 G>A	120	156	Manchester	Sub G>A after Exon 20	AGTGATACCAACAG(G>A)TGAGCAAAAGGACTT	784/788	351
N1303K	21	90	1.80%	Substitute C>G at 36	CATTTAGAAAAA(C>G)TTGGATCCCTATGAAC	788/783	396
N1303H	21	90	rare	Substitute A>C at 34	CATTTAGAAAA(A>C)ACTTTGGATCCCTATGAAC	788/783	396

Table 6

WHAT IS CLAIMED IS:

General tiling claims

1 1. An array of oligonucleotide probes immobilized on a
2 solid support, the array comprising at least two sets of
3 oligonucleotide probes,

4 (1) a first probe set comprising a plurality of
5 probes, each probe comprising a segment of at least three
6 nucleotides exactly complementary to a subsequence of the
7 reference sequence, the segment including at least one
8 interrogation position complementary to a corresponding
9 nucleotide in the reference sequence,

10 (2) a second probe set comprising a corresponding
11 probe for each probe in the first probe set, the corresponding
12 probe in the second probe set being identical to a sequence
13 comprising the corresponding probe from the first probe set or
14 a subsequence of at least three nucleotides thereof that
15 includes the at least one interrogation position, except that
16 the at least one interrogation position is occupied by a
17 different nucleotide in each of the two corresponding probes
18 from the first and second probe sets;

19 wherein the probes in the first probe set have at least
20 two interrogation positions respectively corresponding to each
21 of two contiguous nucleotides in the reference sequence.

1 2. An array of oligonucleotide probes immobilized on a
2 solid support, the array comprising at least four sets of
3 oligonucleotide probes,

4 (1) a first probe set comprising a plurality of
5 probes, each probe comprising a segment of at least three
6 nucleotides exactly complementary to a subsequence of the
7 reference sequence, the segment including at least one
8 interrogation position complementary to a corresponding
9 nucleotide in the reference sequence,

10 (2) second, third and fourth probe sets, each
11 comprising a corresponding probe for each probe in the first
12 probe set, the probes in the second, third and fourth probe
13 sets being identical to a sequence comprising the
14 corresponding probe from the first probe set or a subsequence

15 of at least three nucleotides thereof that includes the at
16 least one interrogation position, except that the at least one
17 interrogation position is occupied by a different nucleotide
18 in each of the four corresponding probes from the four probe
19 sets.

1 3. The oligonucleotide array of claim 2, further
2 comprising a fifth probe set comprising a corresponding probe
3 for each probe in the first probe set, the corresponding probe
4 from the fifth probe set being identical to a sequence
5 comprising the corresponding probe from the first probe set or
6 a subsequence of at least three nucleotides thereof that
7 includes the at least one interrogation position, except that
8 the at least one interrogation position is deleted in the
9 corresponding probe from the fifth probe set.

1 4. The oligonucleotide array of claim 2, further
2 comprising a sixth probe set comprising a corresponding probe
3 for each probe in the first probe set, the corresponding probe
4 from the sixth probe set being identical to a sequence
5 comprising the corresponding probe from the first probe set or
6 a subsequence of at least three nucleotides thereof that
7 includes the at least one interrogation position, except that
8 an additional nucleotide is inserted adjacent to the at least
9 one interrogation position in the corresponding probe from the
10 first probe set.

1 5. The array of claim 2, wherein the first probe set has
2 at least three interrogation positions respectively
3 corresponding to each of three contiguous nucleotides in a
4 reference sequence.

1 6. The array of claim 2, wherein the first probe set has
2 at least 50 interrogation positions respectively corresponding
3 to each of 50 contiguous nucleotides in a reference sequence.

1 7. The array of claim 1 or 2, wherein the first probe
2 set has at least 100 interrogation positions respectively

3 corresponding to each of 100 contiguous nucleotides in a
4 reference sequence.

1 8. The oligonucleotide array of claim 1 or 2, wherein
2 the first probe set has an interrogation position
3 corresponding to each of at least 30% of the nucleotides in a
4 reference sequence and the reference sequence comprises at
5 least 100 nucleotides.

1 9. The oligonucleotide array of claim 8, wherein the
2 first probe set comprises probes which completely span the
3 reference sequence, which probes relative to the reference
4 sequence, overlap one another in sequence.

1 10. The oligonucleotide array of claim 9, wherein the
2 first probe set has an interrogation position corresponding to
3 each of the nucleotides in the reference sequence.

1 11. The oligonucleotide array of claim 10, wherein the
2 probes are oligodeoxyribonucleotides.

1 12. The oligonucleotide array of claim 1 or 2, wherein
2 the array comprises between 100 and 10,000 probes.

1 13. The oligonucleotide array of claim 1 or 2, wherein
2 the array comprises between 10,000 and 100,000 probes.

1 14. The oligonucleotide array of claim 1 or 2, wherein
2 the array comprises between 100,000 and 10,000,000 probes.

1 15. The oligonucleotide array of claim 1 or 2, wherein
2 the probes are linked to the support via a spacer.

1 16. The oligonucleotide array of claim 1 or 2, wherein
2 the segment in each probe of the first probe set that is
3 exactly complementary to the subsequence of the reference
4 sequence is 9-21 nucleotides.

1 17. The oligonucleotide array of claim 16, wherein the
2 segment is n nucleotides long, and the subsequence is at least
3 $n-2$ nucleotides long.

1 18. The oligonucleotide array of claim 1 or 2, wherein
2 each probe of the first probe set consists of the segment that
3 is exactly complementary to the subsequence of the reference
4 sequence.

1 19. The oligonucleotide array of claim 1 or 2, wherein
2 the probes in the second, third and fourth probe sets are
3 identical to the corresponding probe from the first probe set
4 except that the at least one interrogation position is
5 occupied by a different nucleotide in each of the four
6 corresponding probes from the four probe sets.

1 20. The array of claim 2, further comprising fifth,
2 sixth and seventh probe sets, wherein:
3 the segment of each probe in the first set
4 includes at least two interrogation positions each
5 corresponding to a nucleotide in the reference sequence,
6 the second, third and fourth probe sets, each
7 comprise a corresponding probe for each probe in the first
8 probe set, the corresponding probes in the second, third and
9 fourth probe sets being identical to a sequence comprising the
10 corresponding probe from the first probe set or a subsequence
11 of at least three nucleotides thereof that includes a first
12 interrogation position except that the first interrogation
13 position is occupied by a different nucleotide in each of the
14 four corresponding probes from the four probe sets;
15 the fifth, sixth and seventh probe sets, each
16 comprising a corresponding probe for each probe in the first
17 probe set, the probes in the fifth, sixth and seventh probe
18 sets being identical to a sequence comprising the
19 corresponding probe from the first probe set or a subsequence
20 of at least three nucleotides thereof that includes a second
21 interrogation position, except that the second interrogation

22 position is occupied by a different nucleotide in each of the
23 four corresponding probes from the four probe sets.

1 21. The array of claim 2, wherein each probe in the
2 first probe set further comprises a second segment of at least
3 three nucleotides exactly complementary to a second
4 subsequence of the reference sequence, and the probes from the
5 second, third and fourth probe sets comprise the corresponding
6 probe from the first probe set or a subsequence thereof
7 comprising the first and second segments except in the at
8 least one interrogation position.

1 22. The array of claim 2, further comprising:
2 a fifth probe set comprising at least one probe
3 comprising a segment of at least seven nucleotides exactly
4 complementary to a subsequence of the reference sequence
5 except at one or two positions, the segment including at least
6 one interrogation position corresponding to a nucleotide in
7 the reference sequence not at the one or two positions;
8 sixth, seventh and eighth probe sets, each comprising a
9 probe for each probe in the fifth probe set, the corresponding
10 probes from the sixth, seventh & eighth probe sets being
11 identical to a sequence comprising the corresponding probe
12 from the fifth probe set or a subsequence of at least nine
13 nucleotides thereof including the at least one interrogation
14 position and the one or two positions, except in the at least
15 one interrogation position, which is occupied by a different
16 nucleotide in each of the four probes.

1 23. The array of claim 2, wherein the probes are
2 arranged on the substrate so that the first set of probes is
3 arranged in a row across the substrate in an order reflecting
4 the overlap between the probes and the reference sequence, and
5 the additional sets of probes are arranged in columns relative
6 to the probes in said first set, so that probes with the same
7 interrogation position are in the same column and so that each
8 column comprises at least 4 probes.

1 24. The array of Claim 2, wherein said probes are 12 to
2 17 nucleotides in length.

1 25. The array of Claim 2, wherein said probes are 15
2 nucleotides in length and attached by a covalent linkage to a
3 site on a 3'-end of said probes, and said interrogation
4 position is located at position 7, relative to the 3'-end of
5 said probes.

1 26. The array of claim 2, further comprises fifth,
2 sixth, seventh and eighth probe sets,
3 (1) a fifth probe set comprising a plurality of
4 probes, each probe comprising a segment of at least three
5 nucleotides exactly complementary to a subsequence of a second
6 reference sequence, the segment including at least one
7 interrogation position complementary to a corresponding
8 nucleotide in the reference sequence,

9 (2) the sixth, seventh, and eighth probe sets, each
10 comprising a corresponding probe for each probe in the fifth
11 probe set, the probes in the sixth, seventh and eighth probe
12 sets being identical to a sequence comprising the
13 corresponding probe from the fifth probe set or a subsequence
14 of at least three nucleotides thereof that includes the at
15 least one interrogation position, except that the at least one
16 interrogation position is occupied by a different nucleotide
17 in each of the four corresponding probes from the fifth,
18 sixth, seventh and eighth probe sets.

1 27. The array of claim 22, wherein the first, second,
2 third and fourth probe sets have probes of a first length and
3 the fifth, sixth, seventh and eighth probe sets have probes of
4 a second length different from the first length.

Tiling for wildtype and mutant reference sequences

1 28. An array of oligonucleotide probes immobilized on a
2 solid support, the array comprising at least one pair of first
3 and second probe groups, each group comprising a first and
4 second sets of oligonucleotide probes as defined by claim 1;

5 wherein each probe in the first probe set from the
6 first group is exactly complementary to a subsequence of a
7 first reference sequence and each probe in the first probe set
8 from the second group is exactly complementary to a
9 subsequence from a second reference sequence.

1 29. The array of claim 28, wherein the second reference
2 sequence is a mutated form of the first reference sequence.

1 30. The array of claim 28, wherein each group further
2 comprises third and fourth probe sets, each comprising a
3 corresponding probe for each probe in the first probe set, the
4 probes in the second, third and fourth probe sets being
5 identical to a sequence comprising the corresponding probe
6 from the first probe set or a subsequence of at least three
7 nucleotides thereof that includes the interrogation position,
8 except that the interrogation position is occupied by a
9 different nucleotide in each of the four corresponding probes
10 from the four probe sets.

1 31. The array of claim 30 that comprises at least five
2 pairs of first and second probe groups, wherein the probes in
3 the first probe sets from the first groups of the five pairs
4 are exactly complementary to subsequences from five different
5 respective first reference sequences.

1 32. The array of claim 30 that comprises at least forty
2 pairs of first and second probe groups, wherein the probes in
3 the first probe sets from the first groups of the forty pairs
4 are exactly complementary to subsequences from forty
5 respective first reference sequences.

Block tiling

1 33. An array of oligonucleotide probes immobilized on a
2 solid support, the array comprising at least a group of probes
3 comprising:

4 a wildtype probe comprising a segment of at least three
5 nucleotides exactly complementary to a subsequence of a

6 reference sequence, the segment having at least first and
7 second interrogation positions corresponding to first and
8 second nucleotides in the reference sequence,
9 a first set of three mutant probes, each identical to a
10 sequence comprising the wildtype probe or a subsequence of at
11 least three nucleotides thereof including the first and second
12 interrogation positions, except in the first interrogation
13 position, which is occupied by a different nucleotide in each
14 of the three mutant probes and the wildtype probe;
15 a second set of three mutant probes, each identical to a
16 sequence comprising the wildtype probe or a subsequence of at
17 least three nucleotides thereof including the first and second
18 interrogation positions, except in the second interrogation
19 position, which is occupied by a different nucleotide in each
20 of the three mutant probes and the wildtype probe.

1 34. The array of claim 33, wherein the segment of the
2 wildtype probe comprises 3-20 interrogation positions
3 corresponding to 3-20 respective nucleotides in the reference
4 sequence, and the array comprises 3-20 respective sets of
5 three mutant probes, each of the three probes identical to a
6 sequence comprising the wildtype probe or a subsequence
7 thereof including the 3-20 interrogation positions, except
8 that one of the 3-20 interrogation positions is occupied by a
9 different nucleotide in each of the three mutant probes and
10 the wildtype probes, the one of the 3-20 interrogation
11 positions being different in each of the 3-20 respective sets
12 of three mutant probes.

1 35. An array of probes immobilized to a solid support
2 comprising two groups of probes, each group as defined by
3 claim 33, a first group comprising a wildtype probe comprising
4 a segment exactly complementary to a subsequence of a first
5 reference sequence and a second group comprising a wildtype
6 probe comprising a segment exactly complementary to a
7 subsequence of a second reference sequence.

1 36. The array of claim 35, comprising at least 10-100
2 groups of probes, each comprising a wildtype probe comprising
3 a segment exactly complementary to a subsequence of at least
4 10-100 respective reference sequences.

Pooled probes

1 37. A method of comparing a target sequence with a
2 reference sequence, the method comprising:
3 identifying variants of a reference sequence differing
4 from the reference sequence in at least one nucleotide;
5 assigning each variant a designation,
6 providing an array of pools of probes, each pool
7 occupying a separate cell of the array, wherein each pool
8 comprises a probe comprising a segment exactly complementary
9 to each variant sequence assigned a particular designation,
10 contacting the array with a target sequence comprising a
11 variant of the reference sequence;
12 determining the relative hybridization intensities of the
13 pools in the array to the target sequence;
14 determining the target sequence from the relative
15 hybridization intensities of the pools.

1 38. The method of claim 37, wherein the variants are
2 assigned numbers according to an error code.

1 39. The method of claim 37, wherein each variant is
2 assigned a designation having at least one digit and at least
3 one value for the digit, and each pool comprise a probe
4 comprising a segment exactly complementary to each variant
5 sequence assigned a particular value in a particular digit.

1 40. The method of claim 39, wherein the variants are
2 assigned successive numbers in a numbering system of base m
3 having n digits, and the array comprises n x (m-1) pools of
4 probes.

1 41. The method of claim 40, wherein each pool further
2 comprises a probe comprising a segment exactly complementary
3 to the reference sequence.

Trellis tiling

1 42. A pooled probe comprising a segment exactly
2 complementary to a subsequence of a reference sequence except
3 at a first interrogation position occupied by a pooled
4 nucleotide N, a second interrogation position occupied by a
5 pooled nucleotide selected from the group of three consisting
6 of (1) M or K, (2) R or Y and (3) S or W, and a third
7 interrogation position occupied by a second pooled nucleotide
8 selected from the group, wherein the pooled nucleotide
9 occupying the second interrogation position comprises a
10 nucleotide complementary to a corresponding nucleotide from
11 the reference sequence when the second pooled probe and
12 reference sequence are maximally aligned, and the pooled
13 nucleotide occupying the third interrogation position
14 comprises a nucleotide complementary to a corresponding
15 nucleotide from the reference sequence when the third pooled
16 probe and the reference sequence are maximally aligned,
17 wherein N is A, C, G or T(U), K is G or T(U), M is A or C, R
18 is A or G, Y is C or T(U), W is A or T(U) and S is G or C.

1 43. An array of oligonucleotide probes immobilized on
2 solid support, the array comprising:
3 first, second and third cells respectively occupied by
4 first, second and third pooled probes, each pooled probe
5 comprising a segment exactly complementary to a subsequence of
6 a reference sequence except at a first interrogation position
7 occupied by a pooled nucleotide N, a second interrogation
8 position occupied by a pooled nucleotide selected from the
9 group of three consisting of (1) M or K, (2) R or Y and (3) S
10 or W, and a third interrogation position occupied by a second
11 pooled nucleotide selected from the group, wherein the pooled
12 nucleotide occupying the second interrogation position
13 comprises a nucleotide complementary to a corresponding
14 nucleotide from the reference sequence when the pooled probe

15 and the reference sequence are maximally aligned, and the
16 pooled nucleotide occupying the third interrogation position
17 comprises a nucleotide complementary to a corresponding
18 nucleotide from the reference sequence when the pooled probe
19 and the reference sequence are maximally aligned;
20 provided that one of the three interrogation
21 positions in the each of the three pooled probes is aligned
22 with the same corresponding nucleotide in the reference
23 sequence, this interrogation position being occupied by an N
24 in one of the pooled probes, and a different pooled nucleotide
25 in each of the other two pooled probes,
26 wherein N is A, C, G or T(U), K is G or T(U), M is A
27 or C, R is A or G, Y is C or T(U), W is A or T(U) and S is G
28 or C.

1 44. The array of claim 43 further comprising:
2 fourth and fifth cells respectively occupied by fourth
3 and fifth pooled probes, each pooled probe as defined by
4 claim 43,
5 wherein one of the three interrogation position in the
6 second, third and fourth pooled probes is aligned with the
7 same corresponding nucleotide in the reference sequence, this
8 interrogation position being occupied by an N in one of the
9 pooled probes, and a different pooled nucleotide in each of
10 the other two pooled probes,
11 wherein one of the three interrogation position in the
12 third, fourth and fifth pooled probes is aligned with the same
13 corresponding nucleotide in the reference sequence, this
14 interrogation position being occupied by an N in one of the
15 pooled probes, and a different pooled nucleotide in each of
16 the other two pooled probes.

1 45. The array of claim 44, wherein the pooled probes are
2 identical except at the interrogation positions.

1 46. The array of claim 44, wherein the first, second,
2 third, fourth and fifth pooled probes are exactly
3 complementary to five respective subsequences of the reference

4 sequences that from each other by increments of one
5 nucleotide.

Bridge tiling

: 1 47. An array of oligonucleotide probes immobilized on a
- 2 solid support, the array comprising at least four probes:
• 3 a first probe comprising first and second segments, each
: 4 of at least three nucleotides and exactly complementary to
5 first and second subsequences of a reference sequences, the
6 segments including at least one interrogation position
7 corresponding to a nucleotide in the reference sequence,
8 wherein either (1) the first and second subsequences are
9 noncontiguous, or (2) the first and second subsequences are
10 contiguous and the first and second segments are inverted
11 relative to the complement of the first and second
12 subsequences in the reference sequence;
13 second, third and fourth probes, identical to a sequence
14 comprising the first probe or a subsequence thereof comprising
15 at least three nucleotides from each of the first and second
16 segments, except in the at least one interrogation position,
17 which differs in each of the probes.

1 48. The array of claim 47, wherein the first and second
2 subsequences are separated by one or two nucleotides in the
3 reference sequence.

Two interrogation positions (no wildtype)

1 49. An array of oligonucleotide probes immobilized on a
2 solid support, the array comprising at least a set of four
3 probes, each of the probes comprising a segment of at least 7
4 nucleotides that is exactly complementary to a subsequence
5 from a reference sequence, except that the segment may or may
: 6 not be exactly complementary at two interrogation positions,
7 wherein:
: 8 the first interrogation position is occupied by a
9 different nucleotide in each of the four probes,
10 the second interrogation position is occupied by a
11 different nucleotide in each of the four probes,

12 in first and second probes, the segment is exactly
13 complementary to the subsequence, except at not more than one
14 of the interrogation positions, and
15 in third and fourth probes, the segment is exactly
16 complementary to the subsequence, except at both of the
17 interrogation positions.

1 50. An array of probes immobilized to a support, the
2 array comprising at least 100 sets of 4 probes, each set as
3 defined by claim 49, the probes from the at least 100 sets
4 comprising at least 100 respective segments, the segments
5 having at least 100 respective first and second interrogation
6 positions.

Helper mutations

1 51. An array of oligonucleotide probes immobilized on a
2 solid support, the array comprising a set of probes
3 comprising:
4 a first probe comprising a segment of at least 7
5 nucleotides exactly complementary to a subsequence of a
6 reference sequence except at one or two positions, the segment
7 including an interrogation position not at the one or two
8 positions;
9 second, third and fourth mutant probes, each identical to
10 a sequence comprising the wildtype probe or a subsequence
11 thereof including the interrogation position and the one or
12 two positions, except in the interrogation position, which is
13 occupied by a different nucleotide in each of the four probes.

Omission of Perfectly Matched Probe

1 52. An array of oligonucleotide probes immobilized on a
2 solid support, the array comprising at least two sets of
3 oligonucleotide probes,
4 (1) a first probe set comprising a plurality of
5 probes, each probe comprising a segment exactly complementary
6 to a subsequence of at least 3 nucleotides of a reference
7 sequence except at an interrogation position,

8 (2) a second probe set comprising a corresponding
9 probe for each probe in the first probe set, the corresponding
10 probe in the second probe set being identical to a sequence
11 comprising the corresponding probe from the first probe set or
12 a subsequence of at least three nucleotides thereof that
13 includes the interrogation position, except that the
14 interrogation position is occupied by a different nucleotide
15 in each of the two corresponding probes and the complement to
16 the reference sequence,

17 wherein the probes in the first probe set have at
18 least three interrogation positions respectively corresponding
19 to each of three contiguous nucleotides in the reference
20 sequence.

Methods

1 53. A method of comparing a target nucleic acid with a
2 reference sequence comprising a predetermined sequence of
3 nucleotides, the method comprising:

4 (a) hybridizing the target nucleic acid to an array
5 of oligonucleotide probes immobilized on a solid support, the
6 array comprising:

7 (1) a first probe set comprising a plurality of
8 probes, each probe comprising a segment of at least three
9 nucleotides exactly complementary to a subsequence of the
10 reference sequence, the segment including at least one
11 interrogation position complementary to a corresponding
12 nucleotide in the reference sequence,

13 (2) a second probe set comprising a corresponding
14 probe for each probe in the first probe set, the corresponding
15 probe in the second probe set being identical to a sequence
16 comprising the corresponding probe from the first probe set or
17 a subsequence of at least three nucleotides thereof that
18 includes the at least one interrogation position, except that
19 the at least one interrogation position is occupied by a
20 different nucleotide in each of the two corresponding probes
21 from the first and second probe sets;

22 wherein, the probes in the first probe set have at
23 least three interrogation positions respectively corresponding

24 to each of at least three nucleotides in the reference
25 sequence, and
26 (b) determining which probes, relative to one
27 another, in the array bind specifically to the target nucleic
28 acid, the relative specific binding of the probes indicating
29 whether the target sequence is the same or different from the
30 reference sequence.

1 54. The method of claim 53, wherein the array further
2 comprises third and fourth probe sets, each comprising a
3 corresponding probe for each probe in the first probe set, the
4 probes in the second, third and fourth probe sets being
5 identical to a sequence comprising the corresponding probe
6 from the first probe set or a subsequence of at least three
7 nucleotides thereof that includes the at least one
8 interrogation position, except that the at least one
9 interrogation position is occupied by a different nucleotide
10 in each of the four corresponding probes from the four probe
11 sets.

1 55. The method of claim 54, wherein the target sequence
2 has a substituted nucleotide relative to the reference
3 sequence in at least one undetermined position, and the
4 relative specific binding of the probes indicates the location
5 of the position and the nucleotide occupying the position in
6 the target sequence.

1 56. The method of claim 54, wherein:
2 the hybridizing step comprises hybridizing the
3 target nucleic acid and a second target nucleic acid to the
4 array; and
5 the determining step comprises determining which
6 probes, relative to one another, in the array bind
7 specifically to the target nucleic acid or the second target
8 nucleic acid, the relative specific binding of the probes
9 indicating whether the target sequence is the same or
10 different from the reference sequence and whether the second

11 target sequence is the same or different from the reference
12 sequence.

1 57. The method of claim 56, wherein the target sequence
2 has a label and the second target sequence has a second label
3 different from the label.

1 58. The method of claim 56, wherein undetermined first
2 and second proportions of the first and second target
3 sequences are hybridized to the array and the specific binding
4 indicates the proportions.

1 59. The method of claim 54, further comprising:
2 (c) removing the target nucleic acid from the array;
3 (d) hybridizing a second target nucleic acid to the
4 array;
5 (e) determining which probes, relative to one another, in
6 the array bind specifically to the second target nucleic acid,
7 the relative specific binding of the probes indicating whether
8 the second target sequence is the same or different from the
9 reference sequence.

1 60. A method of comparing a target nucleic acid with a
2 reference sequence comprising a predetermined sequence of
3 nucleotides, the method comprising:
4 hybridizing the target sequence to the array of
5 claim 28;
6 determining which probes in the first group,
7 relative to one another, hybridize to the target sequence, the
8 relative specific binding of the probes indicating whether the
9 target sequence is the same or different from the first
10 reference sequence;
11 determining which probes in the second group,
12 relative to one another, hybridize to the target sequence, the
13 relative specific binding of the probes indicating whether the
14 target sequence is the same or different from the second
15 reference sequence.

1 61. The method of claim 60, wherein the hybridizing step
2 comprising hybridizing the target sequence and a second target
3 sequence to the array, and the relative specific binding of
4 the probes from the first group indicates that the target is
5 identical to the first reference sequence, and the relative
6 specific binding of the probes from the second group indicates
7 that the second target sequence is identical to the second
8 reference sequence.

1 62. The method of claim 61, wherein the first and second
2 target sequences are heterozygous alleles of a gene.

Comparative hybridization

1 63. A method of comparing a target nucleic acid with a
2 reference sequence comprising a predetermined sequence of
3 nucleotides, the method comprising:
4 (a) hybridizing the reference sequence to an array
5 of oligonucleotide probes immobilized on a solid support, the
6 array comprising;
7 (1) a first probe set comprising a plurality of
8 probes, each probe comprising a segment of at least 3
9 nucleotides exactly complementary to a subsequence of the
10 reference sequence except in at least one interrogation
11 position;
12 (2) a second probe set comprising a corresponding
13 probe for each probe in the first probe set, the corresponding
14 probe in the second probe set being identical to a sequence
15 comprising the corresponding probe from the first probe set or
16 a subsequence of at least three nucleotides thereof that
17 includes the at least one interrogation position, except that
18 the at least one interrogation position is occupied by a
19 different nucleotide in each of the two corresponding probes
20 from the first and second probe sets; and
21 (b) determining which probes, relative to one
22 another, in the array bind specifically to the reference
23 sequence;
24 (c) hybridizing a target sequence to the array;

25 (d) determining which probes, relative to one
26 another, in the array bind specifically to the target
27 sequence;
28 wherein the relative specific binding of the probes
29 to the reference and the target sequence indicates whether the
30 reference sequence is the same or different from the target
31 sequence.

1 64. The method of claim 63, wherein the reference
2 sequence has a first label and the second reference sequence
3 has a second label different from the first label, and steps
4 (a) and (c) are performed simultaneously.

HIV Chip

1 65. The array of claim 2, wherein the reference sequence
2 is from a human immunodeficiency virus.

1 66. The array of claim 65, wherein the reference
2 sequence is from a reverse transcriptase gene of the human
3 immunodeficiency virus.

1 67. The array of claim 66, wherein the reference
2 sequence is from a protease gene of the human immunodeficiency
3 virus.

1 68. The array of claim 66, wherein the reference
2 sequence is a full-length reverse transcriptase gene.

1 69. The array of claim 68 comprising at least 3200
2 oligonucleotide probes.

1 70. The array of claim 66, wherein the HIV gene is from
2 the BRU HIV strain.

1 71. The array of claim 66, wherein the HIV gene is from
2 the SF2 HIV strain.

1 72. The array of claim 28, wherein the reference
2 sequence is from the coding strand of a reverse transcriptase
3 gene of a human immunodeficiency virus and the second
4 reference sequence is from the noncoding strand of the reverse
5 transcriptase gene.

1 73. The array of claim 28, wherein the first reference
2 sequence is from a reverse transcriptase gene of a human
3 immunodeficiency virus and the second reference sequence
4 comprises a subsequence of the first reference sequence with a
5 substitution of at least one nucleotide.

1 74. The array of claim 73, wherein the substitution
2 confers drug resistance to a human immunodeficiency virus
3 comprising the second reference sequence.

1 75. The array of claim 28, wherein the first and second
2 reference sequences are from a reverse transcriptase gene from
3 first and second strains of a human immunodeficiency virus.

1 76. The array of claim 28, wherein the first reference
2 sequence is from a reverse transcriptase gene of a human
3 immunodeficiency virus and the second reference sequence is
4 from a 16S RNA, or DNA encoding the 16S RNA, from a pathogenic
5 microorganism.

1 77. The array of claim 28, wherein the first reference
2 sequence is from a reverse transcriptase gene of a human
3 immunodeficiency virus and the second reference sequence is
4 from a protease gene of the human immunodeficiency virus.

1 78. The method of claim 54, wherein the reference
2 sequence is from a human immunodeficiency virus.

1 79. The method of claim 78, wherein the reference
2 sequence is from a human immunodeficiency virus and the target
3 sequence is from a second human immunodeficiency virus.

1 80. The method of claim 79, wherein the target sequence
2 has a substituted nucleotide relative to the reference
3 sequence in at least one undetermined position, and the
4 relative specific binding of the probes indicates the location
5 of the position and the nucleotide occupying the position in
6 the target sequence.

1 81. The method of claim 80, wherein the target sequence
2 has a substituted nucleotide relative to the reference
3 sequence in at least one position, the substitution conferring
4 drug resistance to the human immunodeficiency virus, and the
5 relative specific binding of the probes reveals the
6 substitution.

1 82. The method of claim 78, wherein:
2 the hybridizing step comprises hybridizing the
3 target nucleic acid and a second target nucleic acid, the
4 second target sequence being from a reverse transcriptase gene
5 of a third human immunodeficiency virus, to the array; and
6 the determining step comprises determining which
7 probes, relative to one another, in the array bind
8 specifically to the target nucleic acid or the second target
9 nucleic acid, the relative specific binding of the probes
10 indicating whether the target sequence is the same or
11 different from the reference sequence and whether the second
12 target sequence is the same or different from the reference
13 sequence.

1 83. The method of claim 82, wherein the first target
2 sequence has a first label and the second target sequence has
3 a second label different from the first label.

1 84. The method of claim 82, wherein undetermined first
2 and second proportions of the first and second target
3 sequences are hybridized to the array and the specific binding
4 indicates the proportions.

CFTR Chip

1 85. The array of claim 2, wherein the reference sequence
2 is from a CFTR gene.

1 86. The array of claim 85, wherein the reference
2 sequence is exon 10 of a CFTR gene, and said array comprises
3 over 1000 oligonucleotide probes, 10 to 18 nucleotides in
4 length.

1 87. The array of claim 85, wherein said array comprises
2 a set of probes comprising a specific nucleotide sequence
3 selected from the group of sequences comprising:
4 3'-TTTATAXTAG;
5 3'- TTATAGXAGA;
6 3'- TATAGTXGAA;
7 3'- ATAGTAXAAA;
8 3'- TAGTAGXAAC;
9 3'- AGTAGAXACC;
10 3'- GTAGAAXCCA;
11 3'- TAGAAAXCAC; and
12 3'- AGAAACXACA; wherein each set comprises 4 probes,
13 and X is individually A, G, C, and T for each set.

1 88. The array of claim 85, wherein said group of
2 sequences comprises:
3 3'-TTTATAXTAGAAACC;
4 3'- TTATAGXAGAAACCA;
5 3'- TATAGTXGAAACCAC;
6 3'- ATAGTAXAAACCACA;
7 3'- TAGTAGXAACCACAA;
8 3'- AGTAGAXACCACAAA;
9 3'- GTAGAAXCCACAAAG;
10 3'- TAGAAAXCACAAAGG; and
11 3'- AGAAACXACAAAGGA; wherein each set comprises 4
12 probes, and X is individually A, G, C, and T for each set.

1 89. The array of claim 32, wherein the forty first
2 reference sequences are from a CFTR gene.

1 90. The array of claim 89, wherein each of the forty
2 first reference sequences includes a site of a mutation and at
3 least one adjacent nucleotide.

1 91. The array of claim 90, wherein each of the forty
2 first reference sequences comprises at least five contiguous
3 nucleotides from a CFTR gene.

1 92. The array of claim 89, wherein at least one first
2 reference sequence is a from the coding strand of the cystic
3 fibrosis gene and at least one first reference sequence is
4 from the noncoding strand of the CFTR gene.

1 93. An array of oligonucleotide probes immobilized on a
2 solid support, the array comprising at least a group of probes
3 comprising:

4 a wildtype probe exactly complementary to a subsequence
5 of a reference sequence from a cystic fibrosis gene, the
6 segment having at least five interrogation positions
7 corresponding to five contiguous nucleotides in the reference
8 sequence,

9 a first set of three mutant probes, each identical to the
10 wildtype probe, except in a first of the five interrogation
11 positions, which is occupied by a different nucleotide in each
12 of the three mutant probes and the wildtype probe;

13 a second set of three mutant probes, each identical to
14 the wildtype probe, except in a second of the five
15 interrogation positions, which is occupied by a different
16 nucleotide in each of the three mutant probes and the wildtype
17 probe;

18 a third set of three mutant probes, each identical to the
19 wildtype probe, except in a third of the five interrogation
20 positions, which is occupied by a different nucleotide in each
21 of the three mutant probes and the wildtype probe;

22 a fourth set of three mutant probes, each identical to
23 the wildtype probe, except in a fourth of the five
24 interrogation positions, which is occupied by a different

25 nucleotide in each of the three mutant probes and the wildtype
26 probe;

27 a fifth set of three mutant probes, each identical to the
28 wildtype probe, except in a fifth of the five interrogation
29 positions, which is occupied by a different nucleotide in each
30 of the three mutant probes and the wildtype probe.

1 94. The array of claim 93 comprising first and second
2 groups of probes, each group as defined by claim 93, the first
3 group comprising a wildtype probe exactly complementary to a
4 first reference sequence, and the second group comprising a
5 wildtype probe exactly complementary to a second reference
6 sequence, wherein the second reference sequence is a mutated
7 form of the first reference sequence.

1 95. The array of claim 94, wherein the first reference
2 sequence is from a CFTR gene and the second reference sequence
3 is a mutated form of the first reference sequence.

1 96. The method of claim 56, wherein the target sequence
2 and the second target sequence are from heterozygous alleles
3 of a CFTR gene.

P53 Chip

1 97. The array of claim 2, wherein the reference sequence
2 is a sequence from a p53 gene.

1 98. The array of claim 2, wherein the reference sequence
2 is from an hMLH1 gene.

1 99. The array of claim 2, wherein the reference sequence
2 is from an MSH2 gene.

1 100. The array of claim 28, wherein the reference
2 sequence is from a human P53 gene and the second reference
3 sequence is from an hMLH1 gene.

1 101. The array of claim 100, further comprising:

2 ninth, tenth, eleventh and twelfth probe sets,

3 (1) the ninth probe set comprising a plurality of
4 probes, each probe comprising a segment of at least three
5 nucleotides exactly complementary to a subsequence of a third
6 reference sequence, the segment including at least one
7 interrogation position complementary to a corresponding
8 nucleotide in the third reference sequence,

9 (2) the tenth, eleventh and twelfth probe sets,
10 each comprising a corresponding probe for each probe in the
11 ninth probe set, the probes in the tenth, eleventh and twelfth
12 probe sets being identical to a sequence comprising the
13 corresponding probe from the ninth probe set or a subsequence
14 of at least three nucleotides thereof that includes the at
15 least one interrogation position, except that the at least one
16 interrogation position is occupied by a different nucleotide
17 in each of the four corresponding probes from the ninth,
18 tenth, eleventh and twelfth probe sets.

1 102. The array of claim 97, wherein the first probe set
2 has at least 60 interrogation positions corresponding to at 60
3 contiguous nucleotides from exon 6.

1 103. The array of claim 98, wherein the reference
2 sequence is exon 5 of a p53 gene, the probes are 17
3 nucleotides long, and the first set of probes is exactly
4 complementary to the reference sequence, and the at least one
5 interrogation position is at position 7, relative to a 3'-end
6 of each probe, which 3'-end is covalently attached to the
7 substrate.

Mitochondrial Chip

1 104. The array of claim 2, wherein the reference
2 sequence is from a mitochondrial genome.

1 105. The array of claim 104, wherein said reference
2 sequence is a sequence of a D-loop region.

1 106. The array of claim 105, wherein D-loop region is
2 full-length.

1 107. The array of claim 104, wherein said reference
2 sequence is at least 90% of a full-length mitochondrial
3 genome.

1 108. The array of claim 104, wherein the reference
2 sequence is bounded by positions 16280 to 356 of the
3 mitochondrial genome.

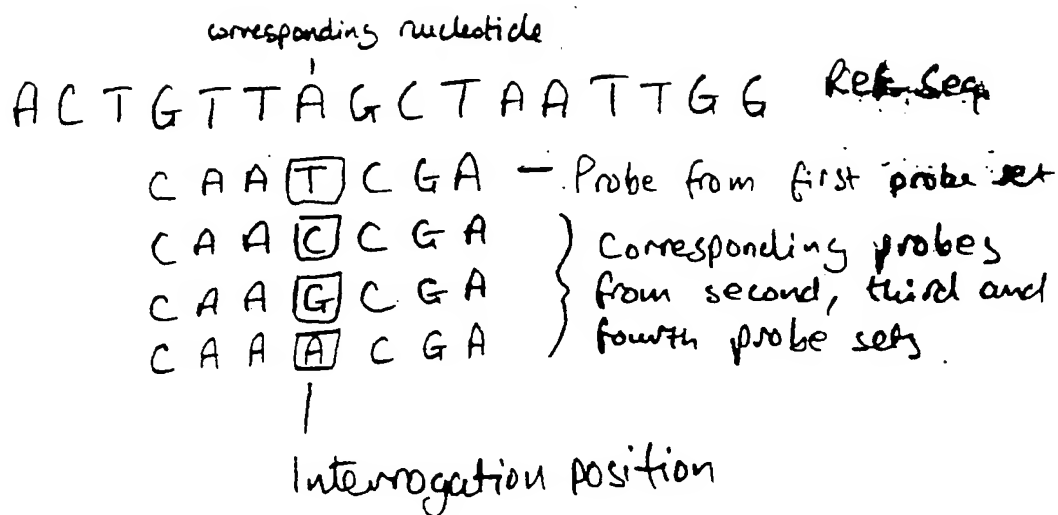


Fig. 1

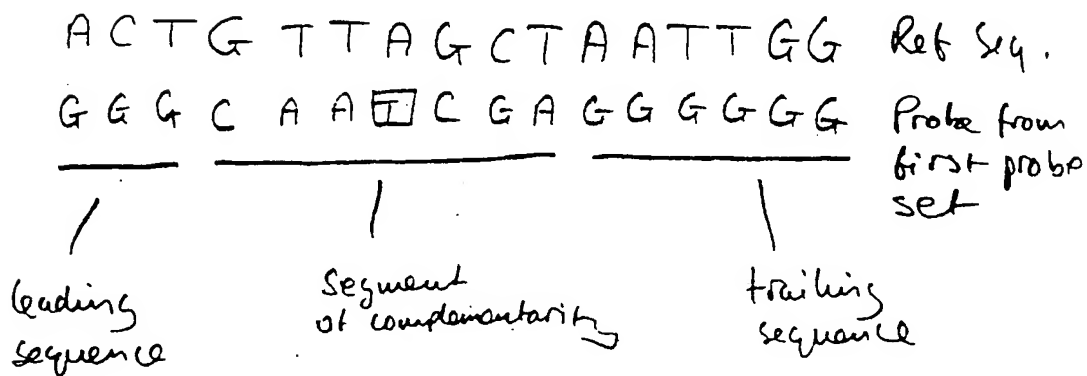


Fig. 2

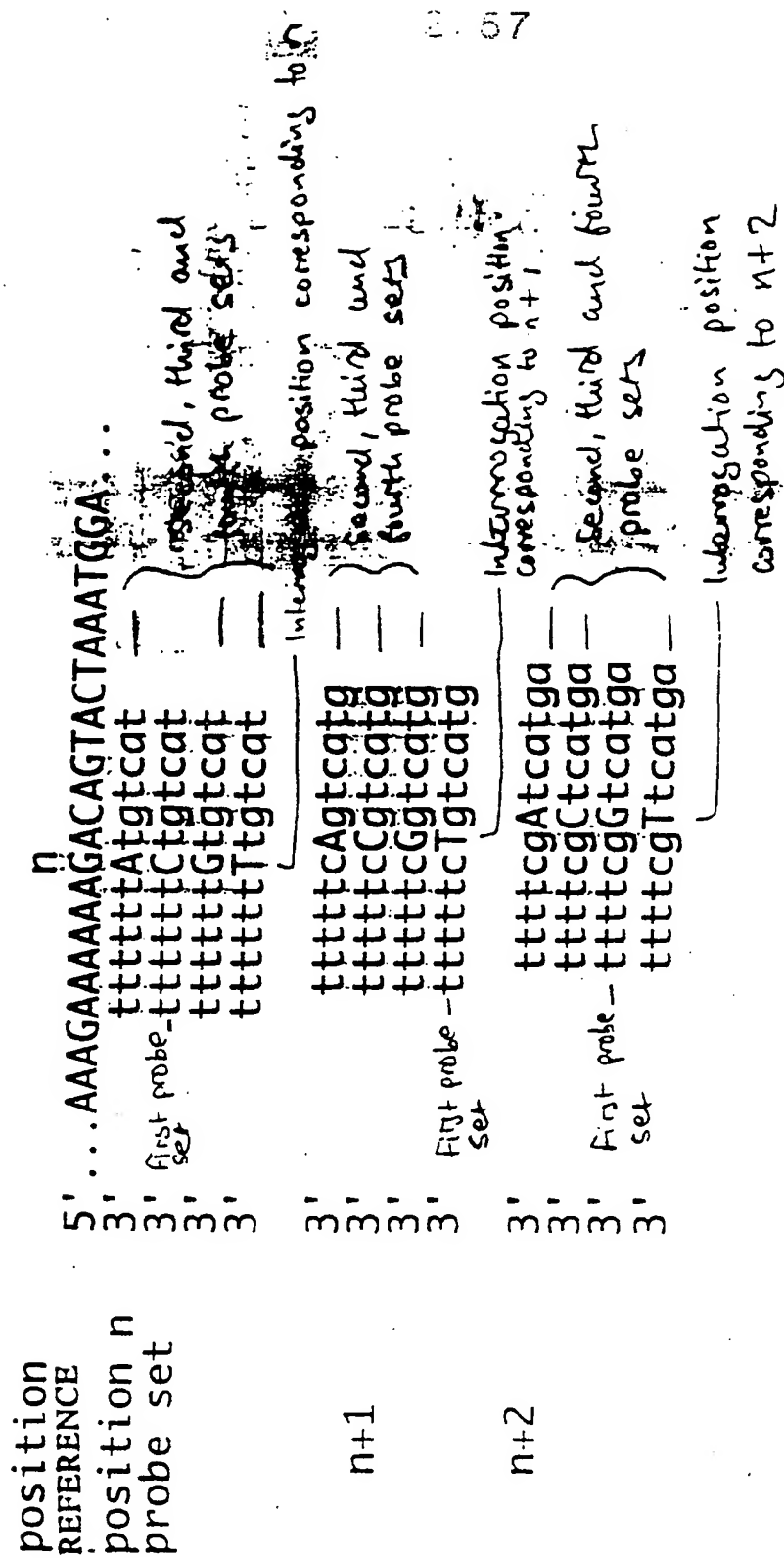


Fig. 3

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Fig. 5

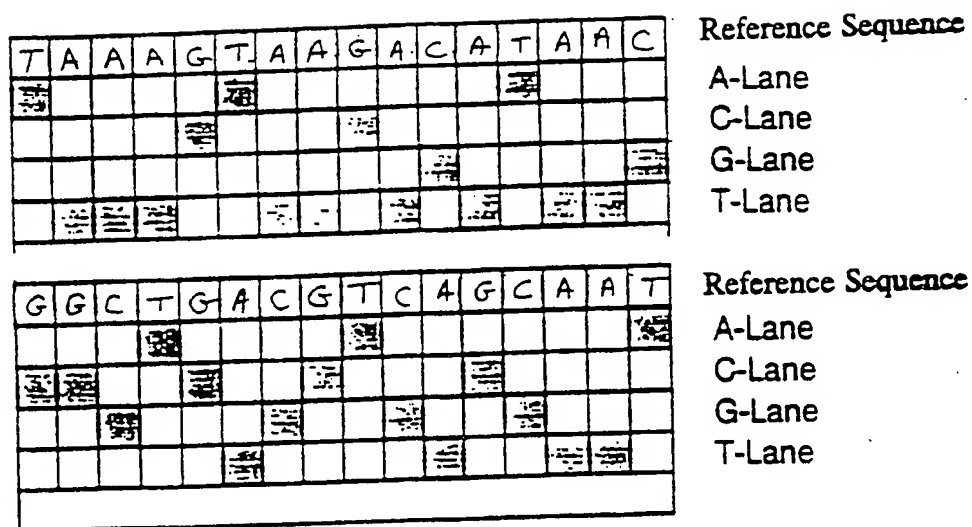


FIG. 5 : Tiled Array with Probes for the Detection of Point Mutations

3' - CCGACTACAGTCGTT
 3' - CCGACTCCAGTCGTT
 3' - CCGACTGCAGTCGTT
 3' - CCGACTTCAGTCGTT

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n corresponding nucleotide
 A C T G T T A G C T A A T T G G Ref. Seq.
 C A A T C G A - Probe from first set
 C A A C G A [T] - Deletion probe
 C A A T A C G [A] } Insertion
 C A A T C C G [A] } Probes
 C A A T G C G [A]
 C A A T T C G [A]

Fig. 6

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n_1 n_2 n_3 Corresponding nucleotides
 A C T G \overline{T} T A G C T A A T T G G Ref. Seq.

C \overline{A} A \overline{T} C \overline{G} A Probe from first set
 I_1 I_2 I_3 Interrogation positions

C \overline{C} A T C G A
 C \overline{G} A T C G A
 C \overline{T} A T C G A
 I_1

} Corresponding probes from second, third and fourth probe sets

C A A \overline{A} C G A
 C A A \overline{C} C G A
 C A A \overline{G} C G A
 I_2

} Corresponding probes from fifth, sixth and seventh probe sets

C A A T C \overline{A} A
 C A A T C \overline{C} A
 C A A T C \overline{T} A
 I_3

} Corresponding probes from eighth, ninth and tenth probe sets

Fig. 7

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n_3 n_4 n_1 n_2
 A C T G T T A G C T A A T T G G Ref. Seq.

C A A T C A A T
 C A C T C C A T
 C A G T C G A T
 C A T T C T A T

I_1 I_2 Interrogation positions

T G A C T A T
 T G C C G A T
 T G G C C A T
 T G T C A A T

I_3 I_4 Interrogation positions

Fig. 8

n corresponding nucleotide
 A T T C C C G G G A T C

A G G G C C A T — Probe from first probe set
 A G G C C C A T
 A G G A C C A T
 A G G T C C A T } corresponding probes from second, third and fourth probe sets

— helps mutation

Interrogation

position

Fig. 9

3157

HV 407A

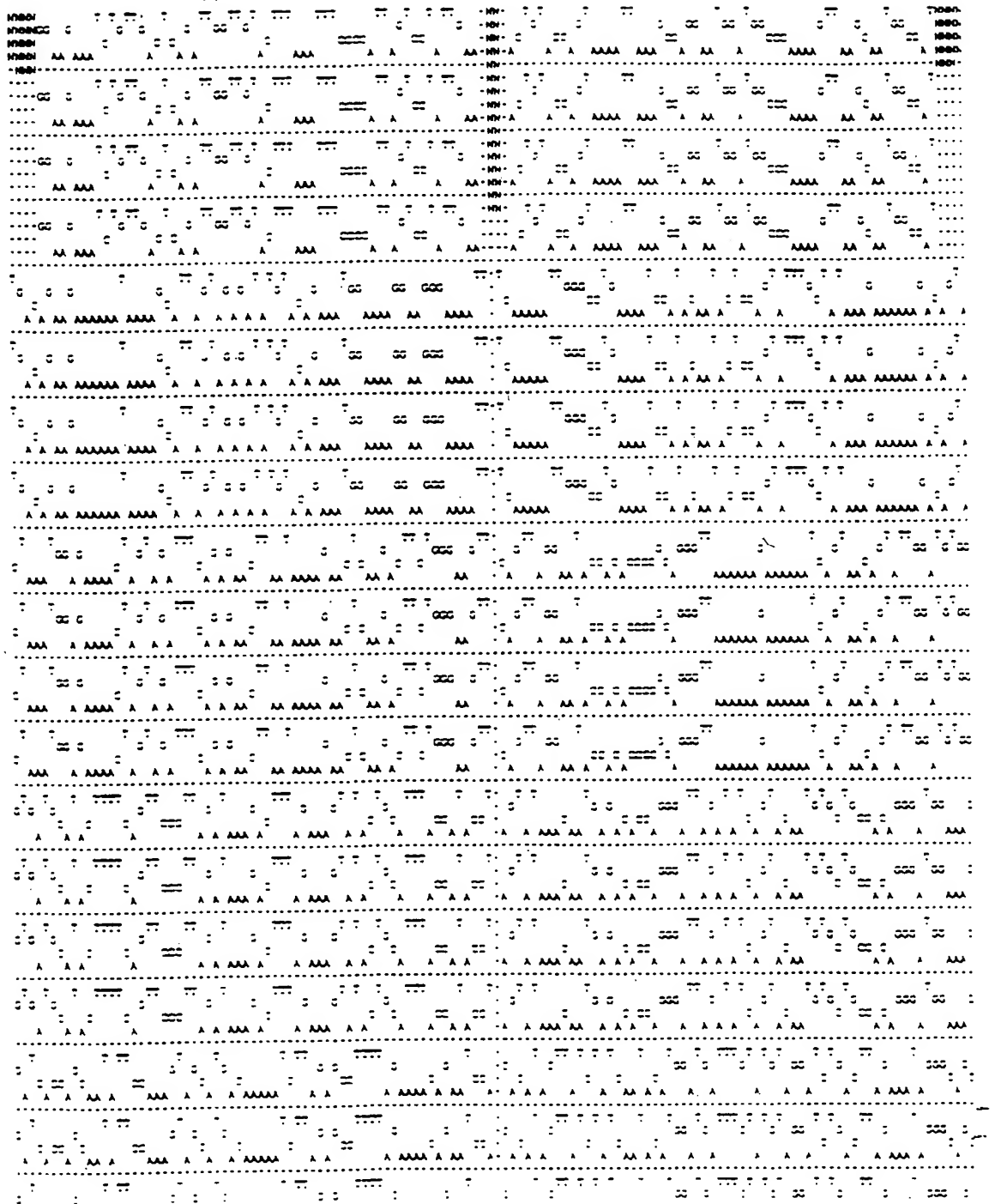
130 x 140

13/7

15/9

17/9

19/10

Fig. 10
Page 1 of 2

9757

HV787A (2)

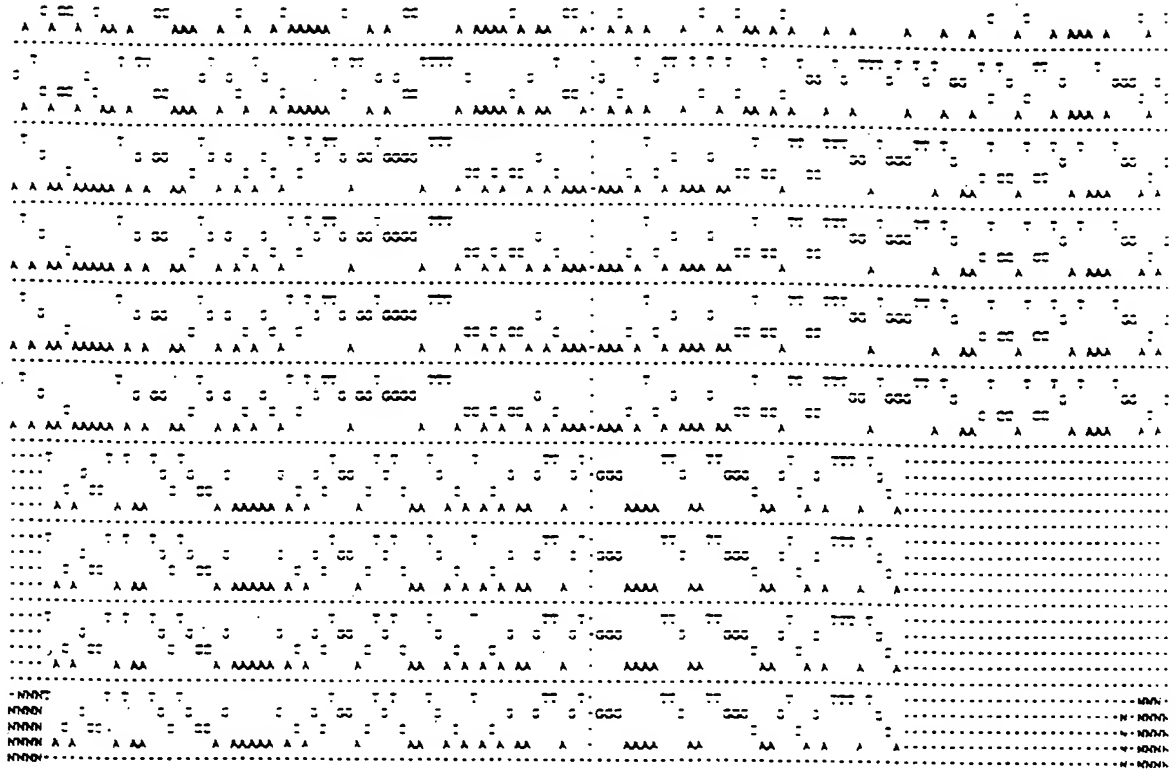
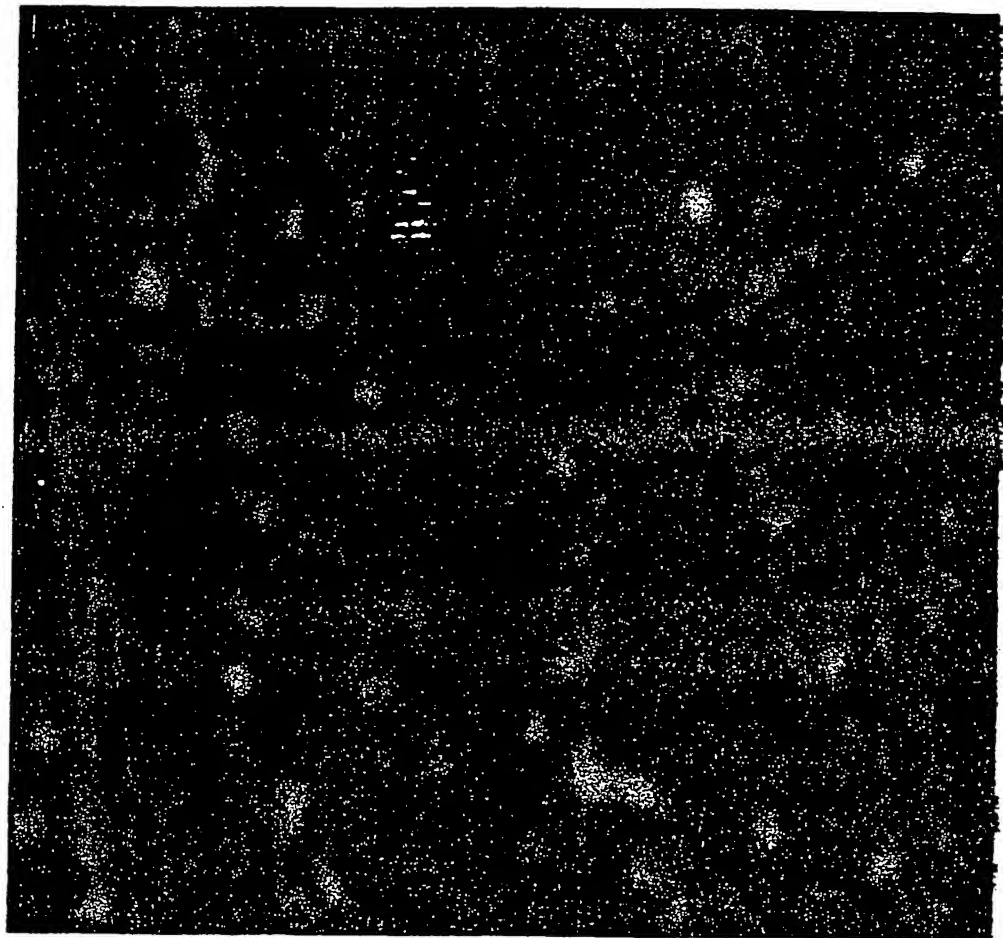


Fig. 10
Page 2 of 2

10.57



13 probe length
15
17
19

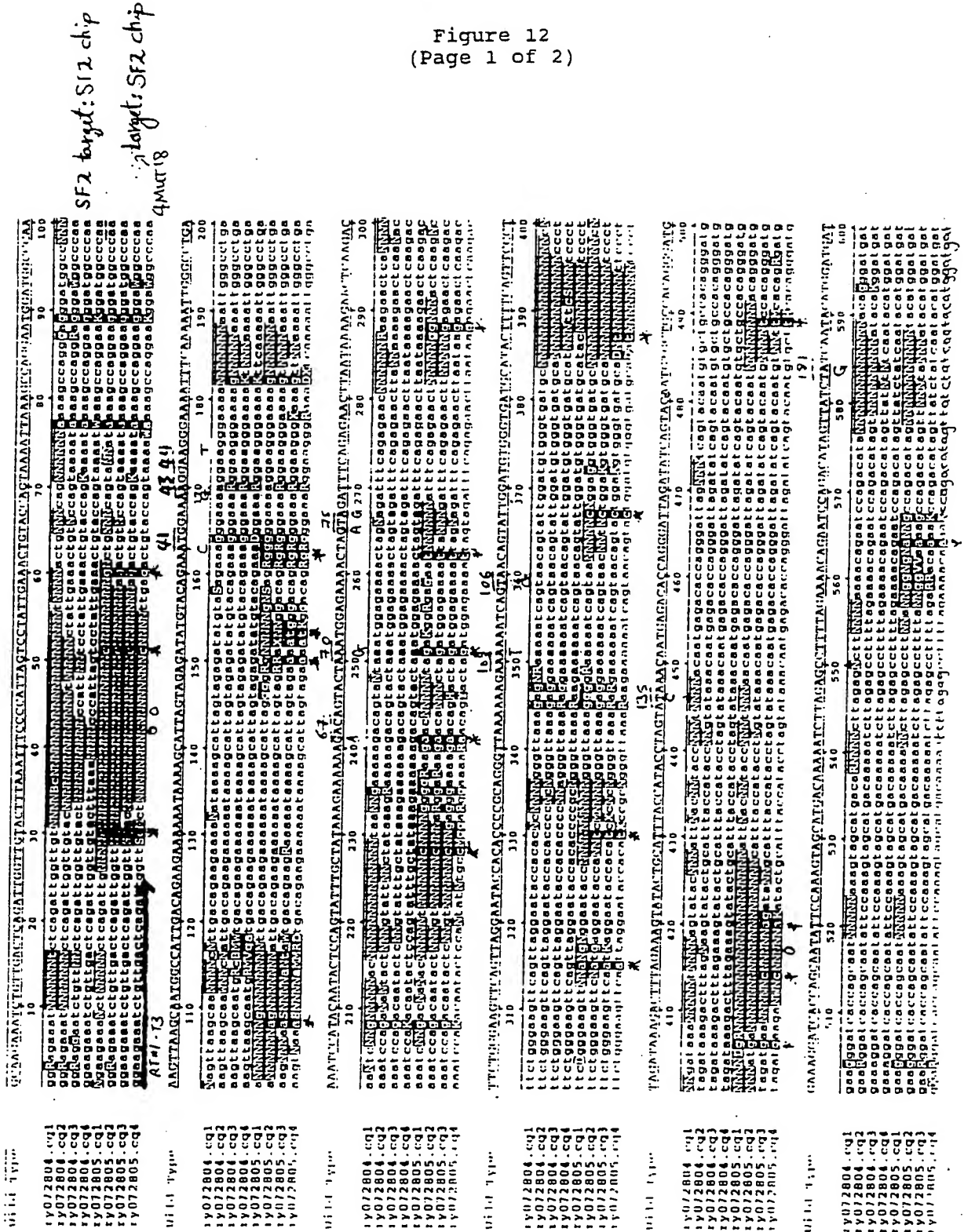
MC07060:

= 407 water chip hybridized with fragmented pPol 19 RNA

Fig. 11

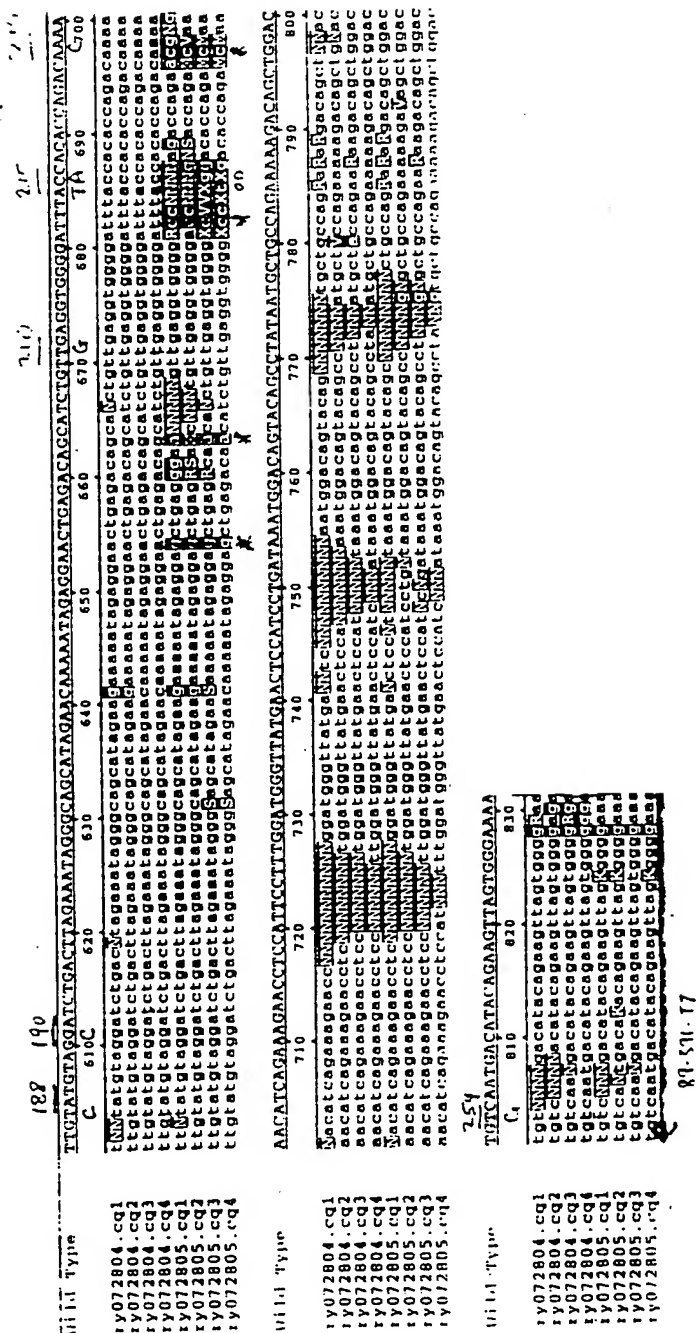
11/57

Figure 12
(Page 1 of 2)



12157

Figure 12
(Page 2 of 2)



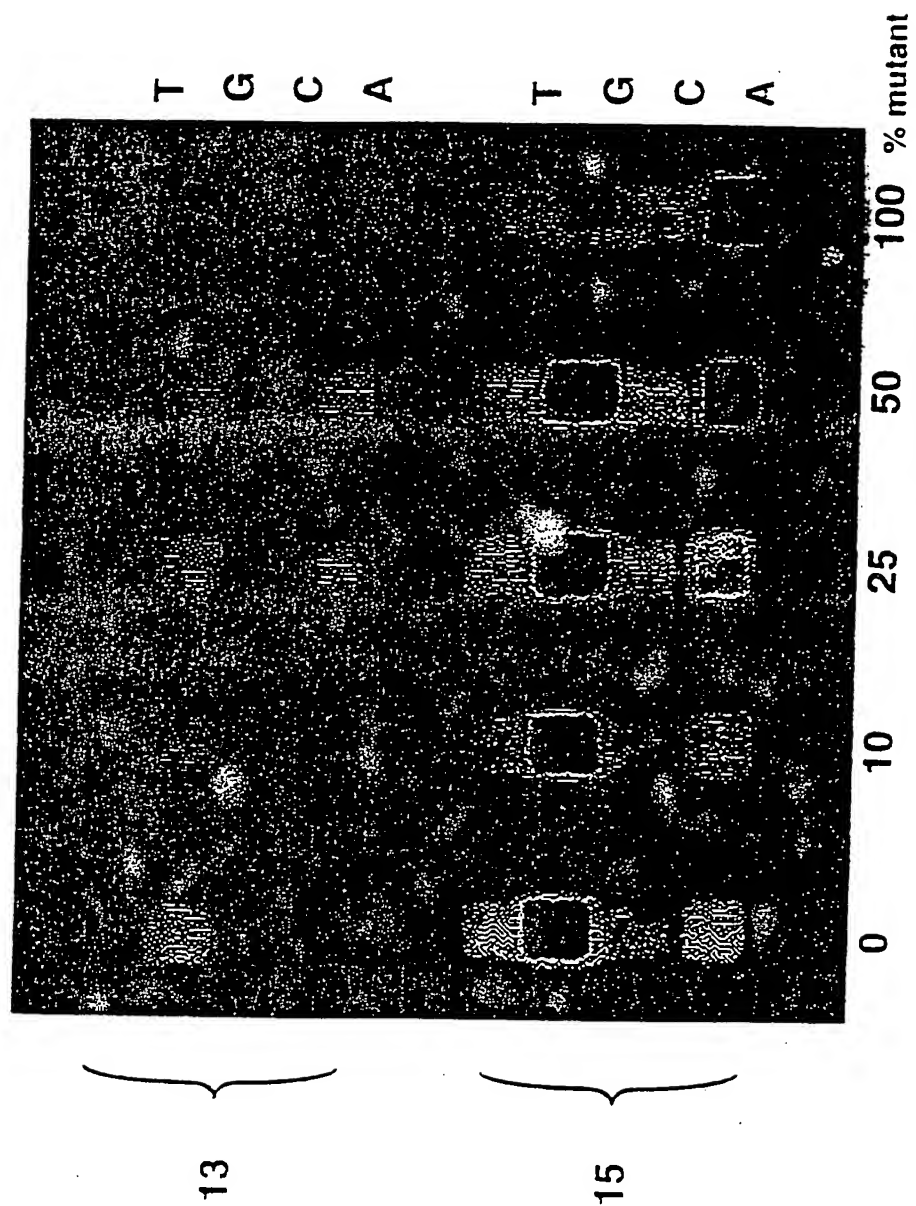
13/57

5'Fluorescein-AAAGAAAAAGACAGTAAATGGAGAAAAT	wildtype
PROBE 3' tttttt•tgtcat	13mers
PROBE 3' ctttttt•tgtcatg	15mers
PROBE 3' tctttttt•tgtcatga	17mers
PROBE 3' ttctttttt•tgtcatgat	19mers
5'Fluorescein-AAAGAAAAAAACAGTAAATGGAGAAAAT	mutant

Fig. 13

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Fig. 14



15:57

Genotyping of HIV-1 Protease Gene

14 pre and post-add I treated Patients

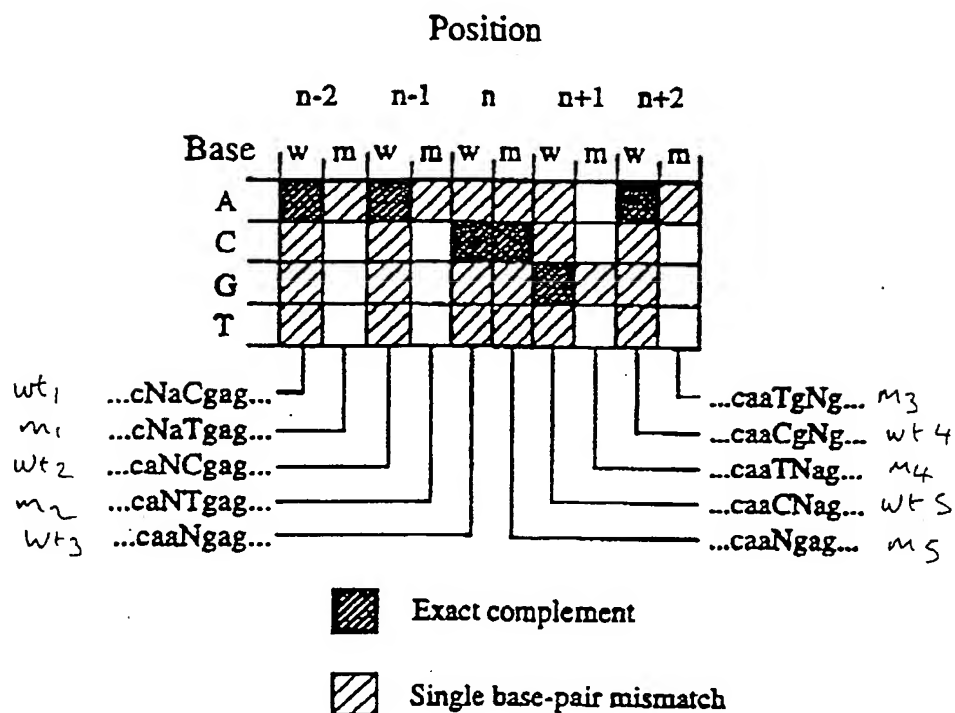
[illegible][illegible]

Fig. 15

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Array Design for the R553X Point Mutation

Wild-Type Pattern



Wild-Type Sequence: 5'-AGGTCAA**C**GAGCAA-3'

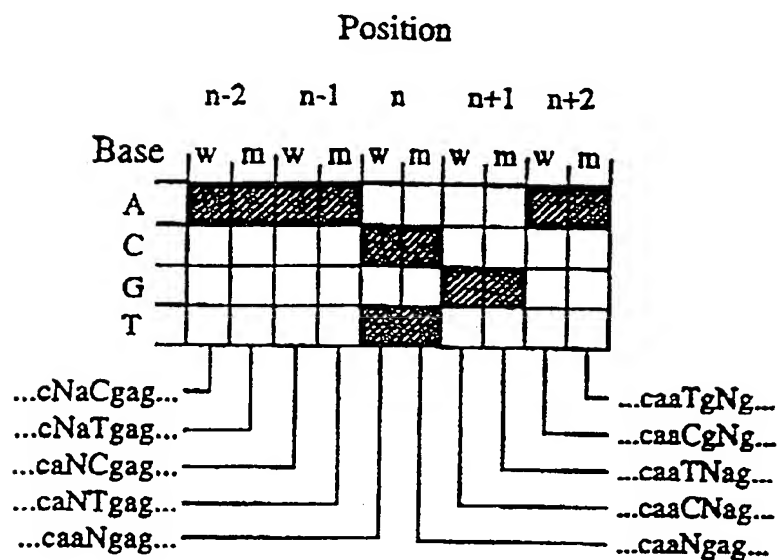
Mutant Sequence: 5'-AGGTCAA**T**GAGCAA-3'

Fig. 16

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Array Design for the R553X Point Mutation

Heterozygote Pattern



Wild-Type Sequence: 5'-AGGTCAACGAGCAA-3'

Mutant Sequence: 5'-AGGTCAATGAGCAA-3'

Fig. 17

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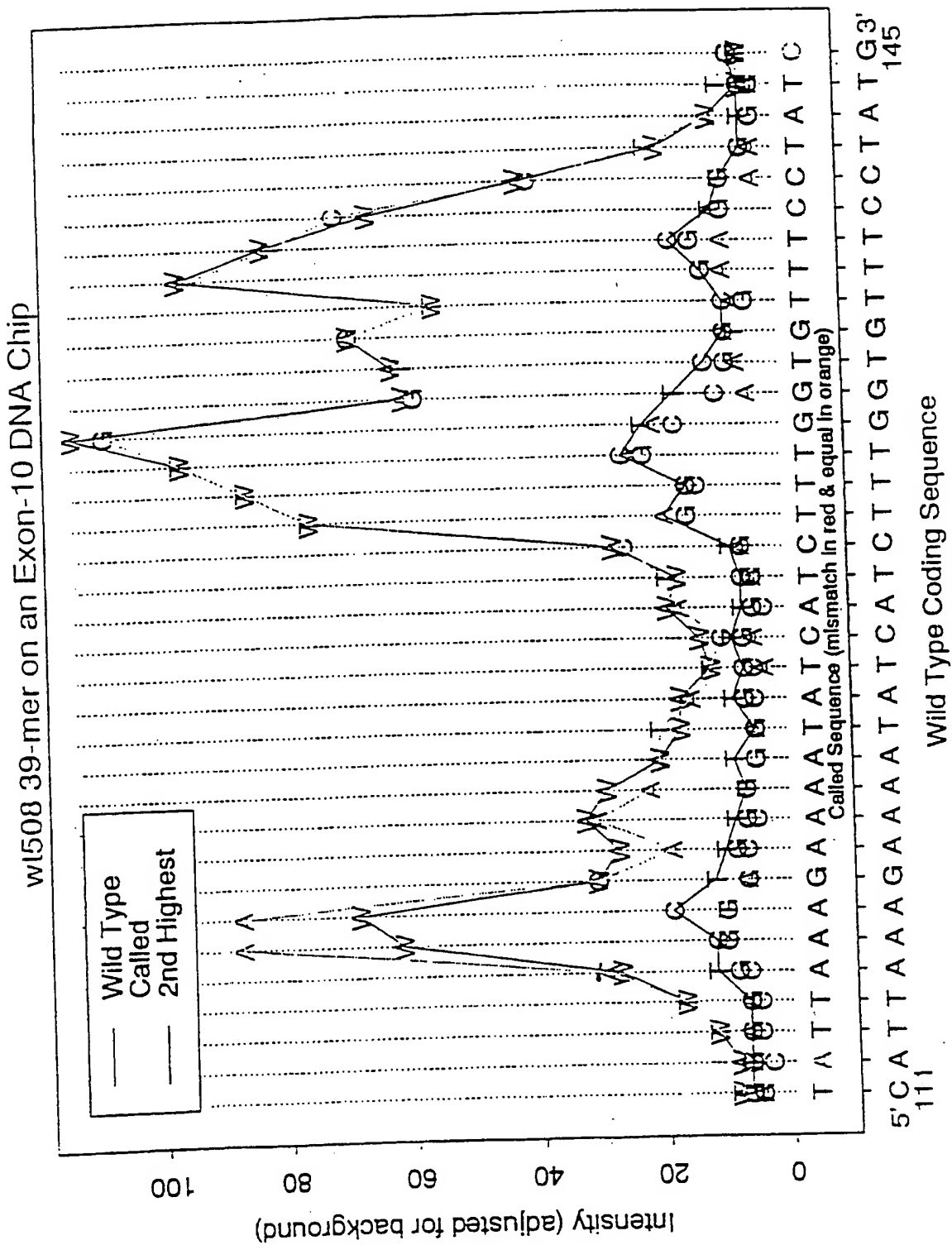


Fig. 19
Page 1 of 3

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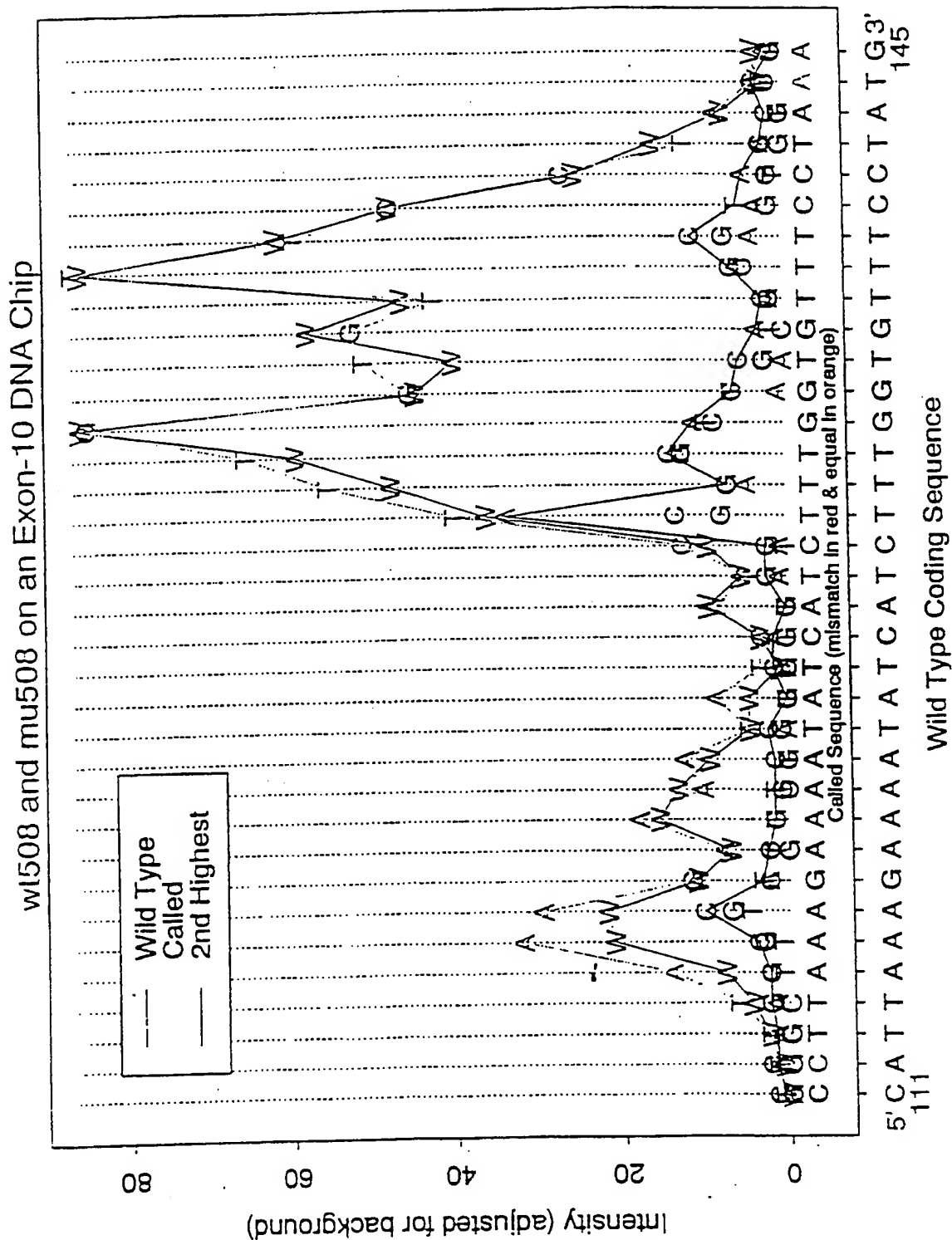


Fig. 19
Page 2 of 3

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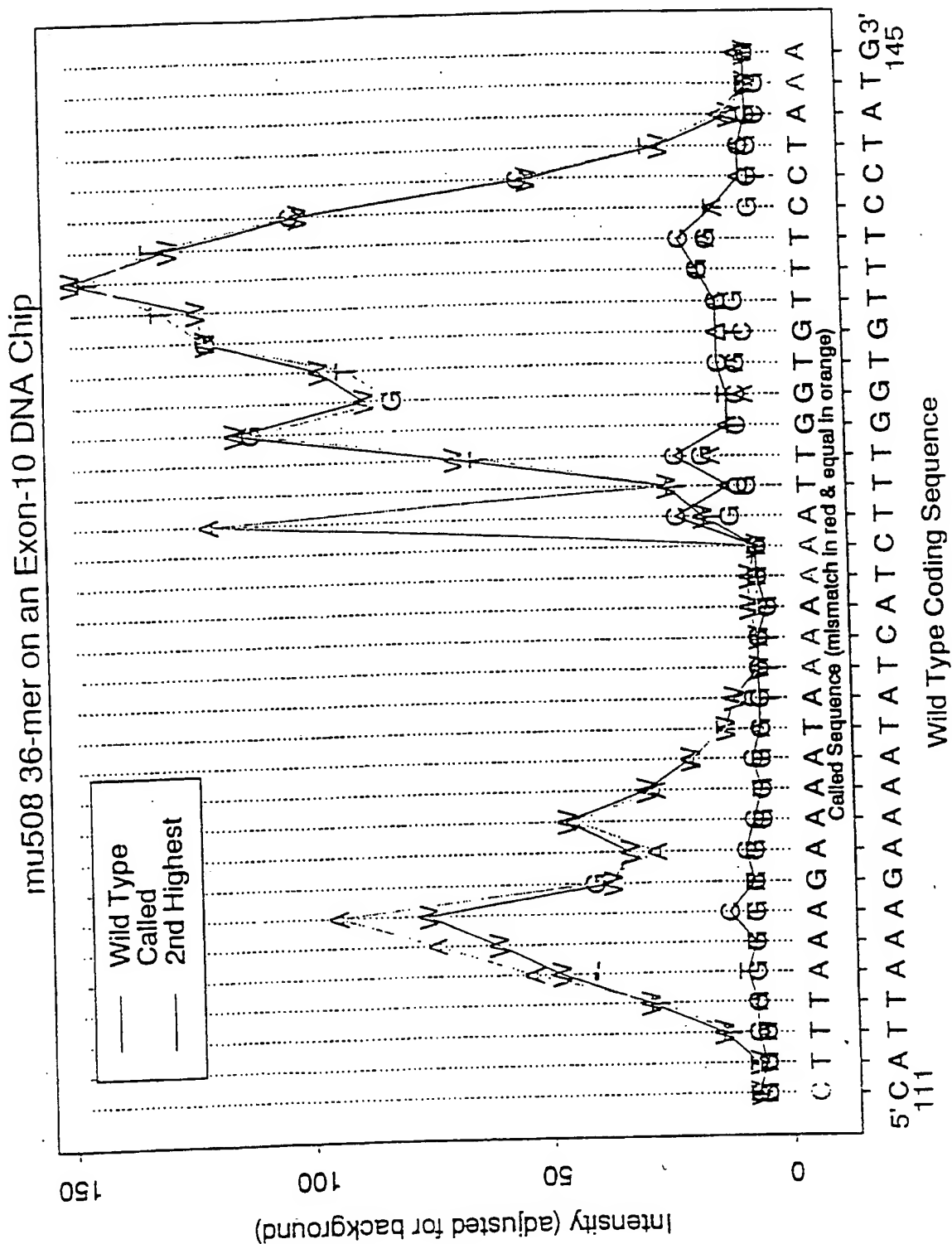


Fig. 19
Page 3 of 3

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Probe Sequence
Wild-Type Lane
A-Lane
C-Lane
G-Lane
T-Lane
Target Sequence

GGAGTCTCCCATTTAATT



A

5'-CCTTCAGAGGTAATAATTAA



B

5'-CCTTCAGAGGTAATAATTAA



C

5'-CCTTCAGAGGTAATAATTAA

Fig. 20

23/07

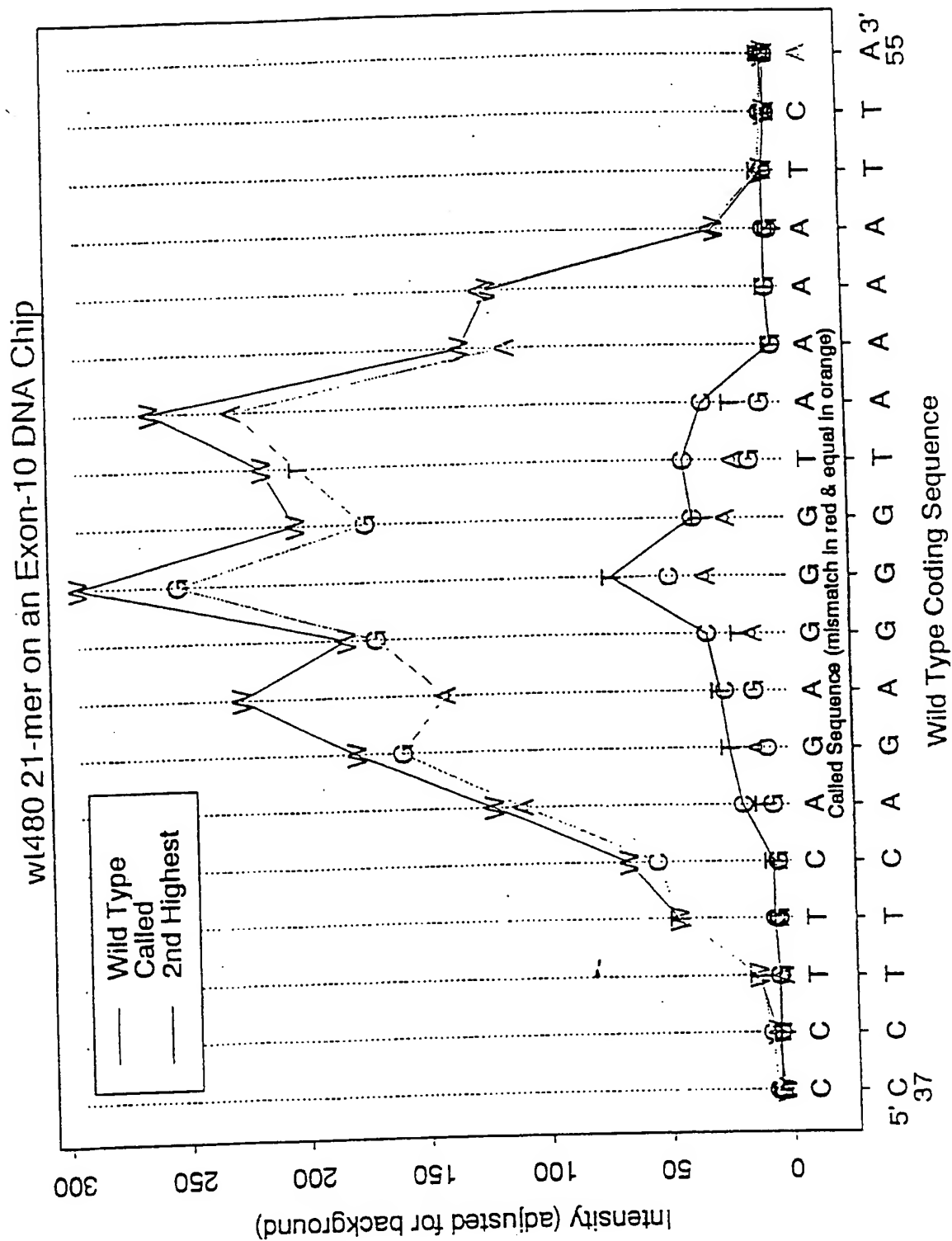


Fig. 21
Page 1 of 3

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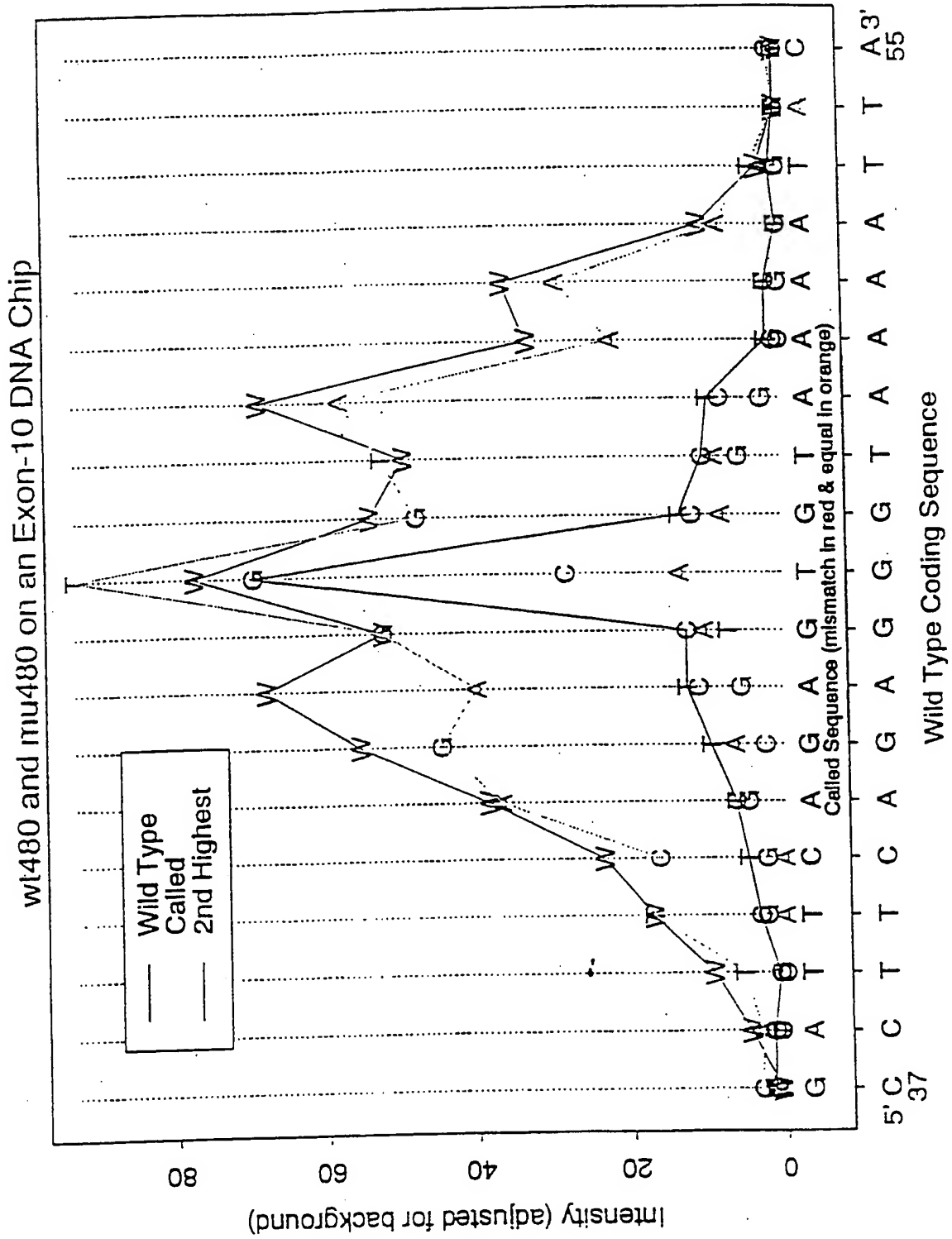


Fig. 21
Page 2 of 3

mu480 21-mer on an Exon-10 DNA Chip

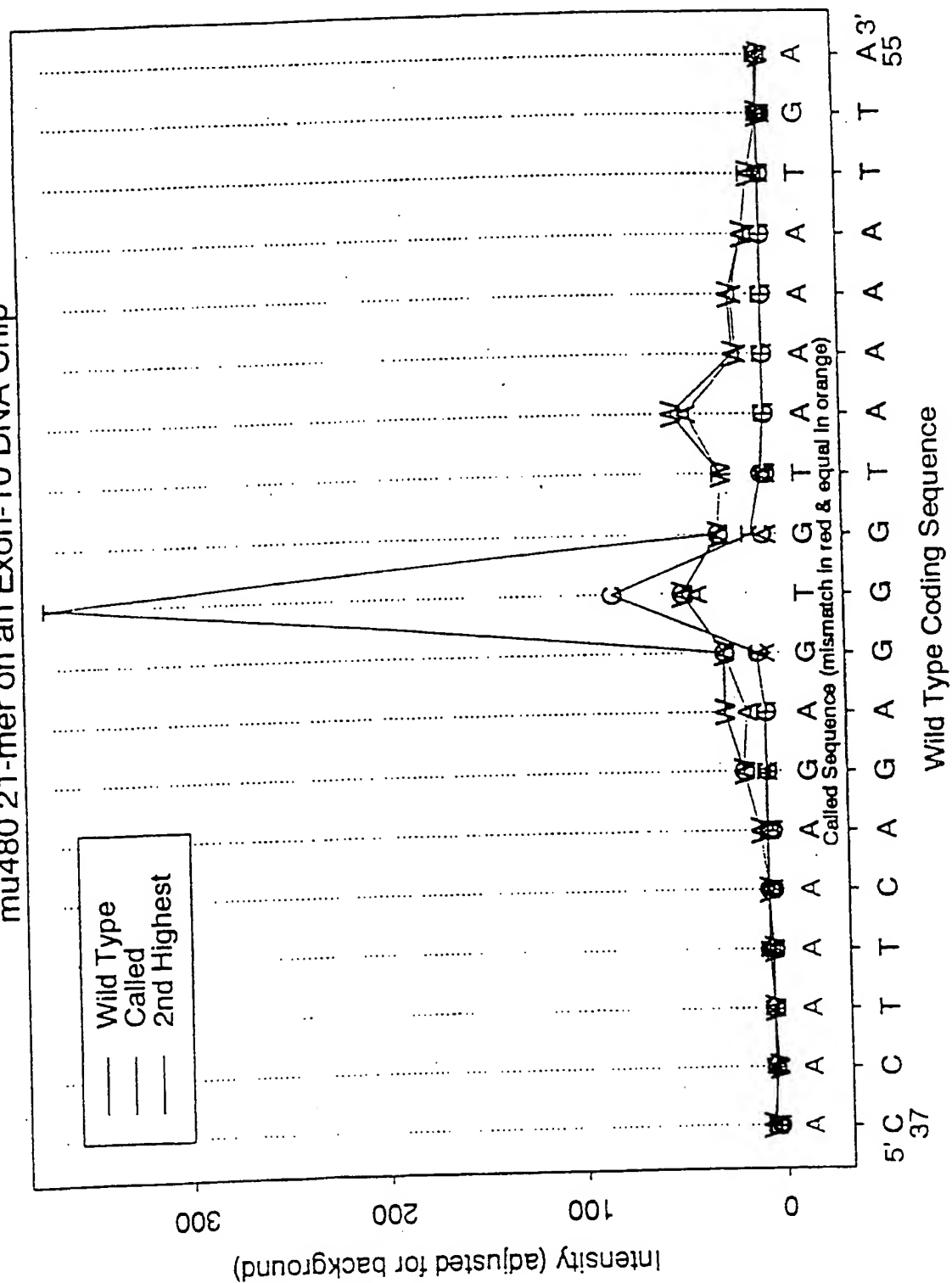


Fig. 21
Page 3 of 3

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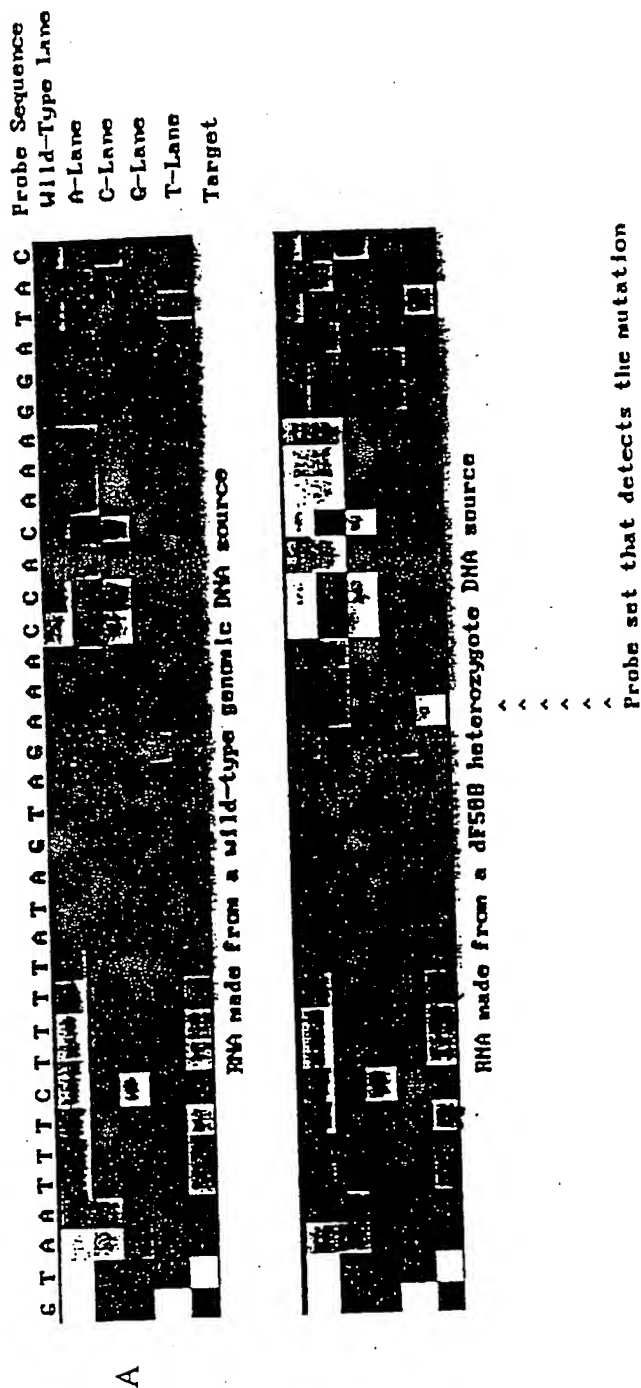


Fig. 22

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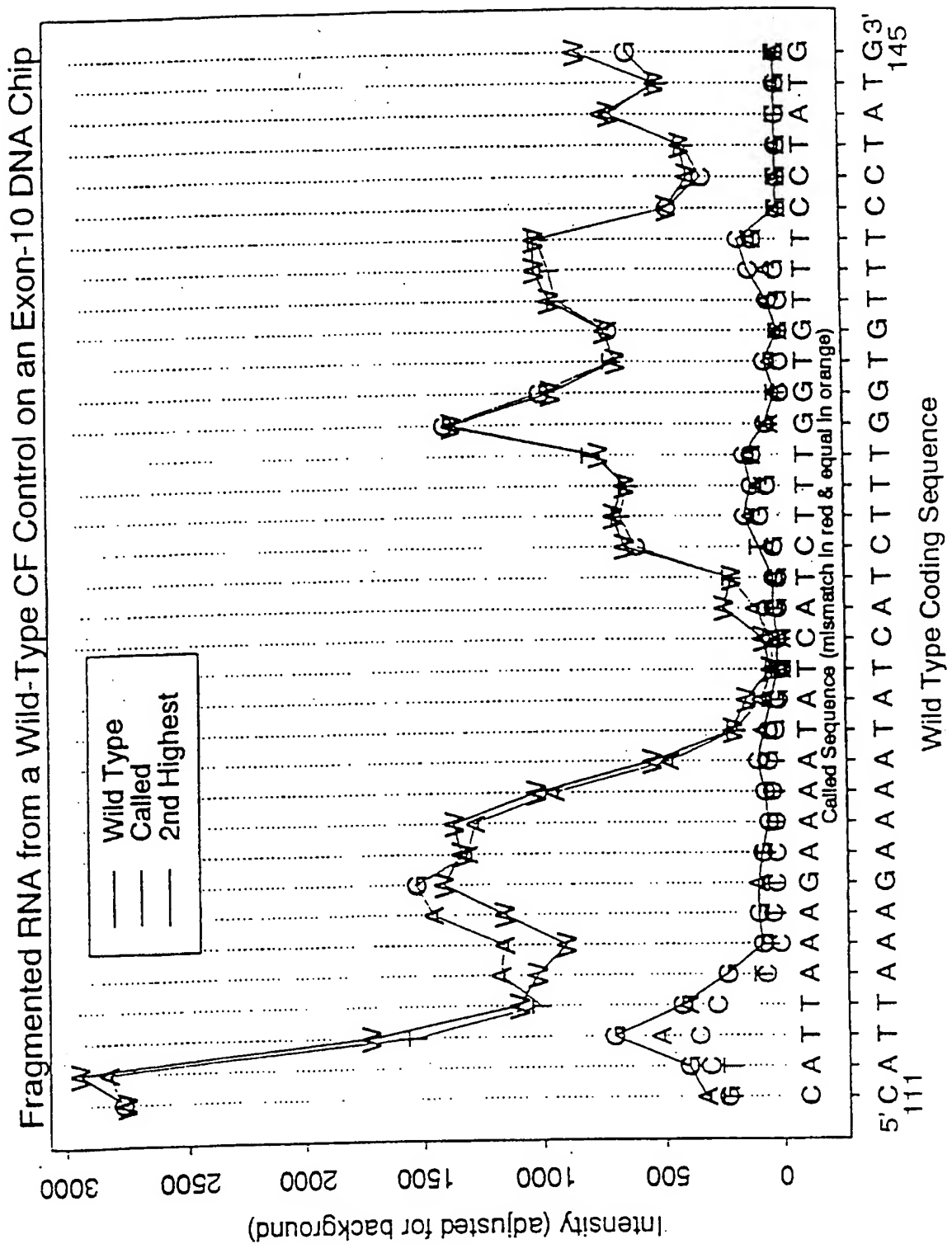


Fig. 23
Page 1 of 2

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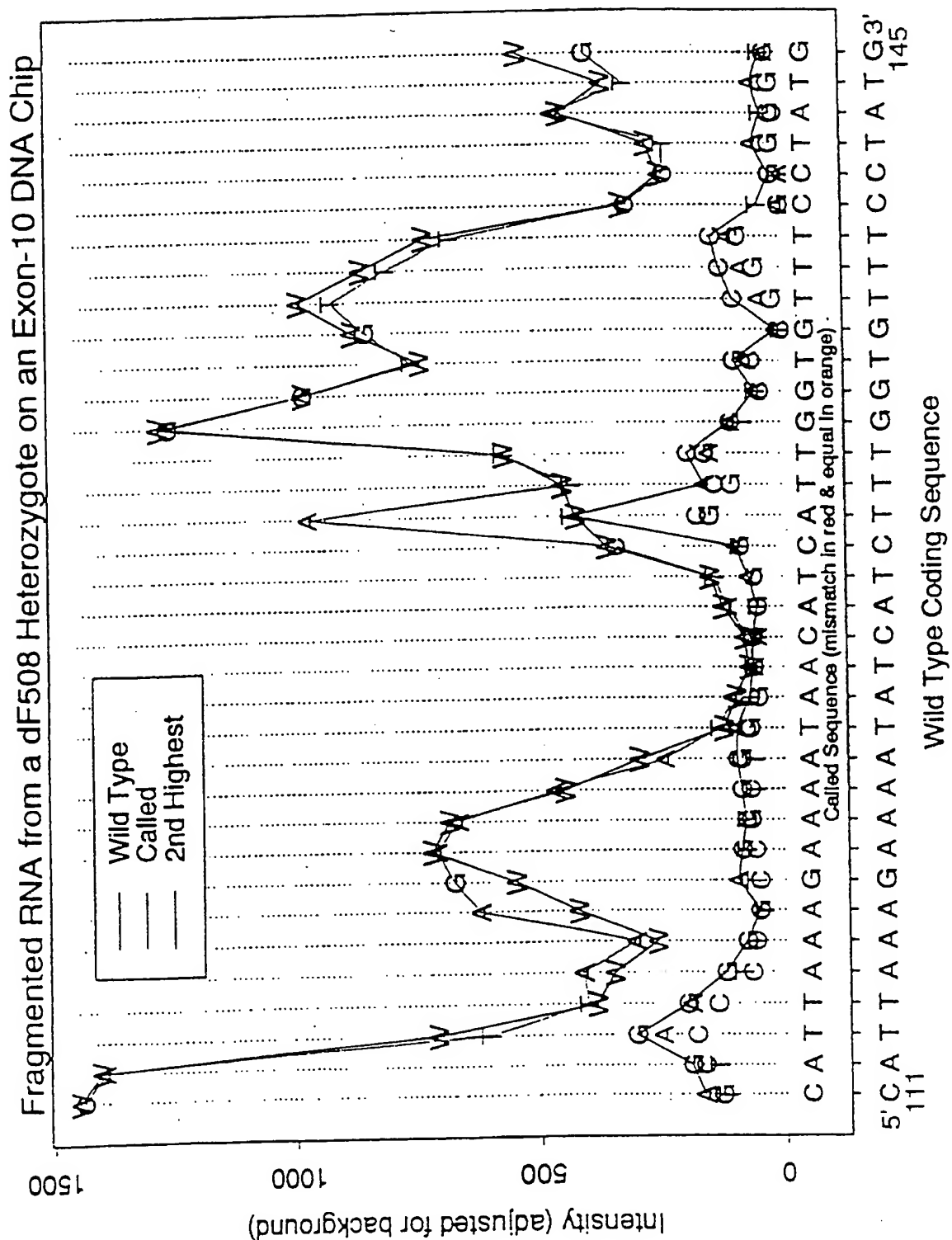


Fig. 23

20/57.

A

B

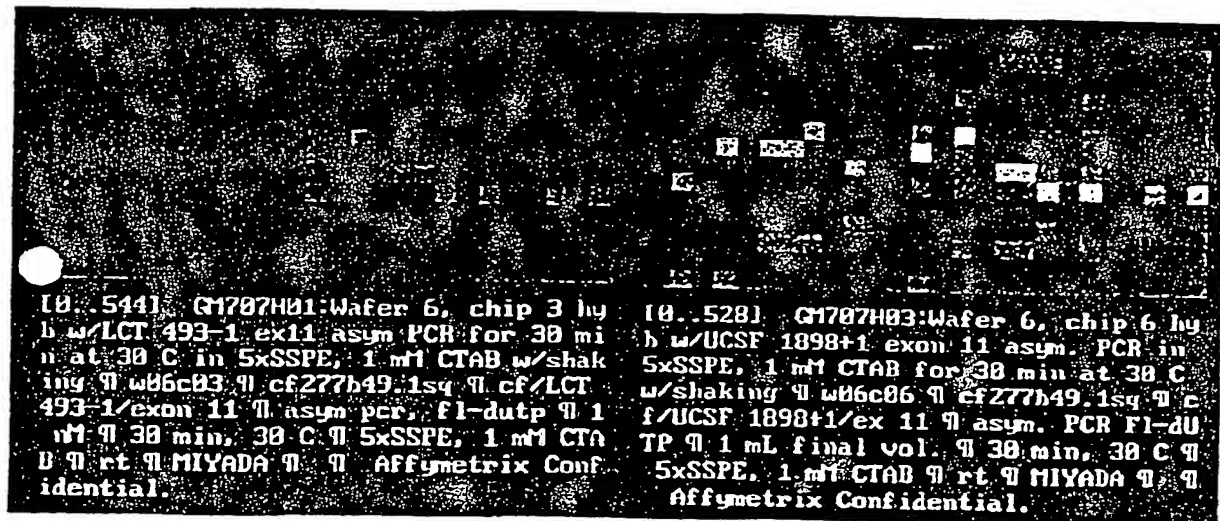


Fig. 24

80.17

A



Fig. 25
Page 1 of 2

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B

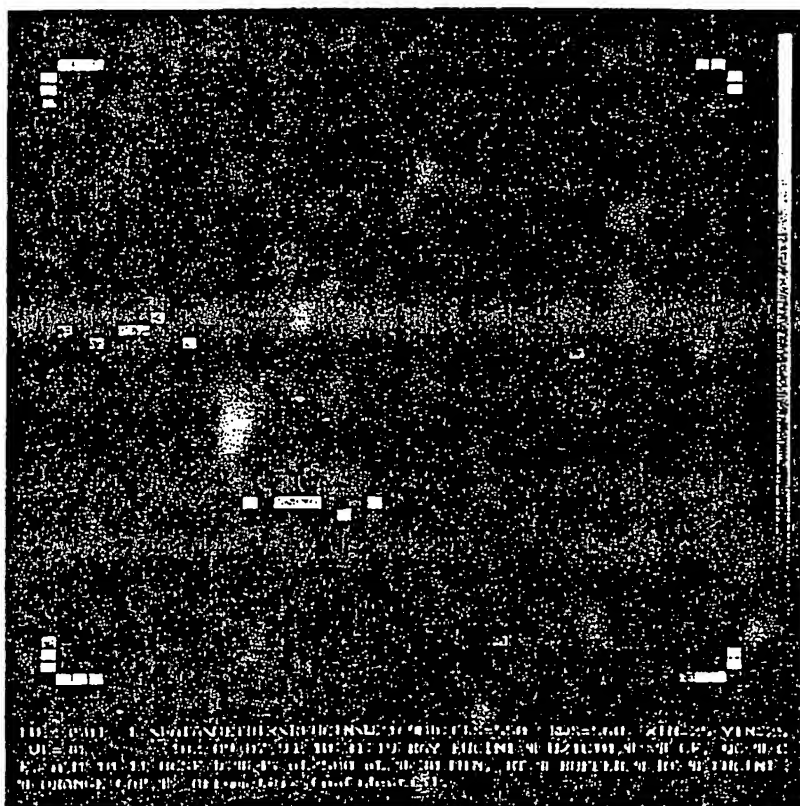


Fig. 25
Page 2 of 2

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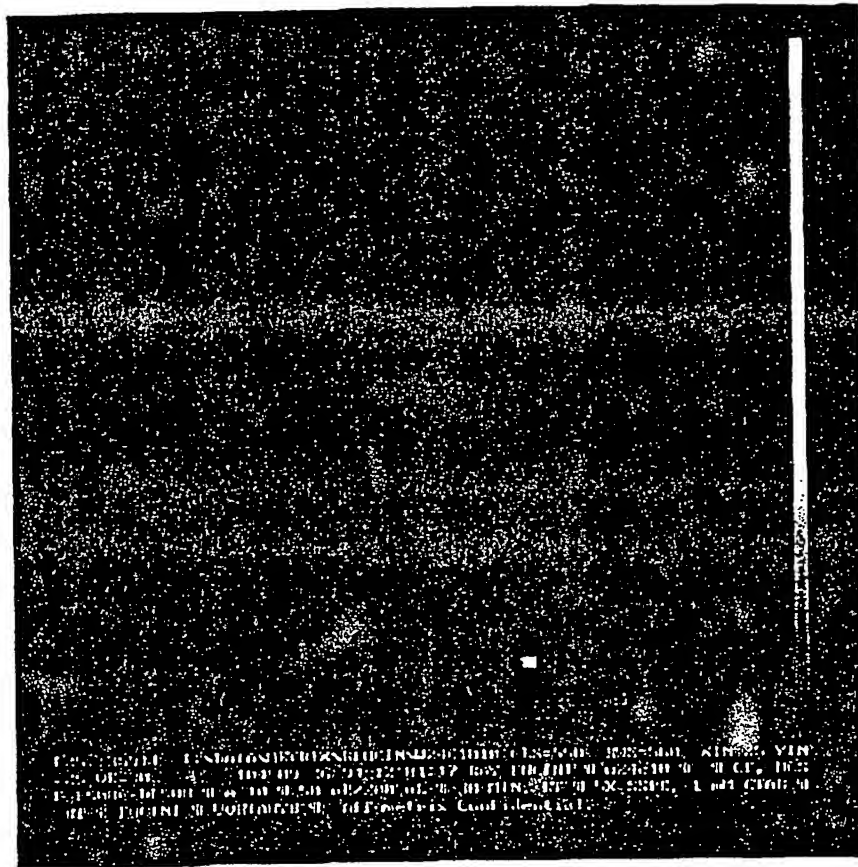


Fig. 26

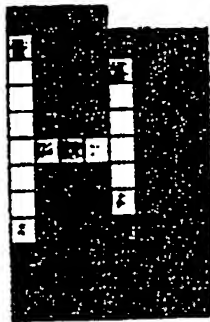
P53 EXON 6 CODON 192 REGION: 12MER PROBES

[illegible]

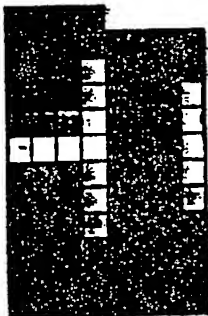
Detection of 12-mer One-Base Substitution P53 Targets

Fig. 29

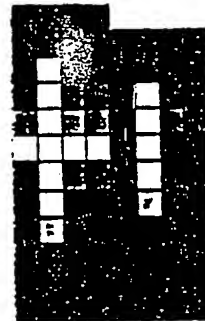
WT ("G" Substitution)
Target 12-mer



"A" Substitution 12-mer Target



"T" Substitution Target 12-mer

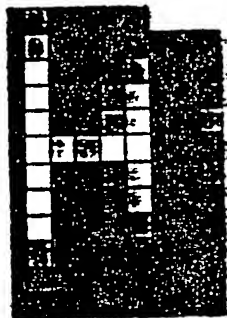


"C" Substitution Target 12-mer



Fig. 31

4:1 Mixture of WT and
"A" Substitution 12-mer
Targets



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Figs. 29 and 31

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P53 EXON 6 CODON 192 REGION

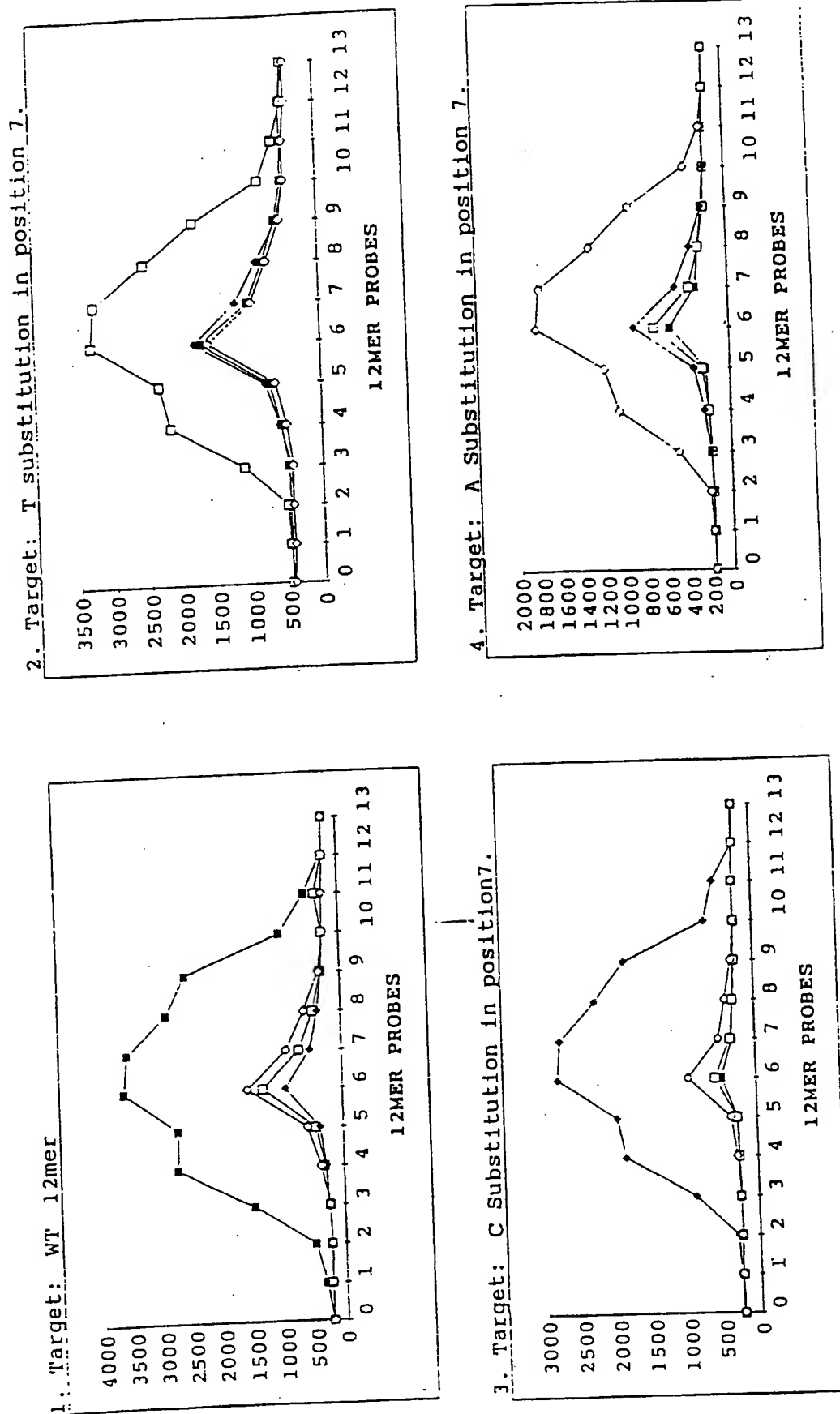


Fig. 30

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153 EXON 6 CODON 192 REGION

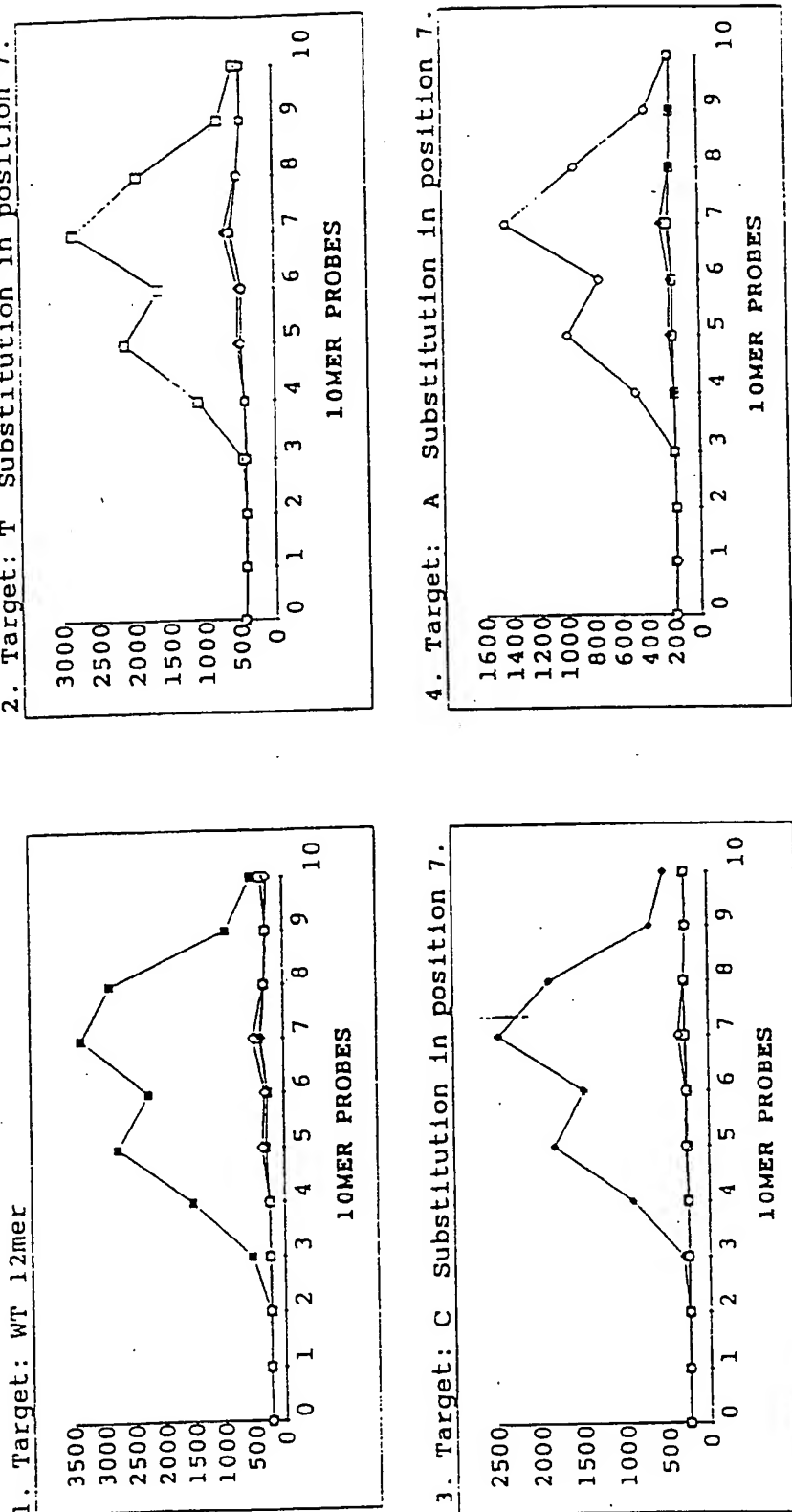


Fig. 32

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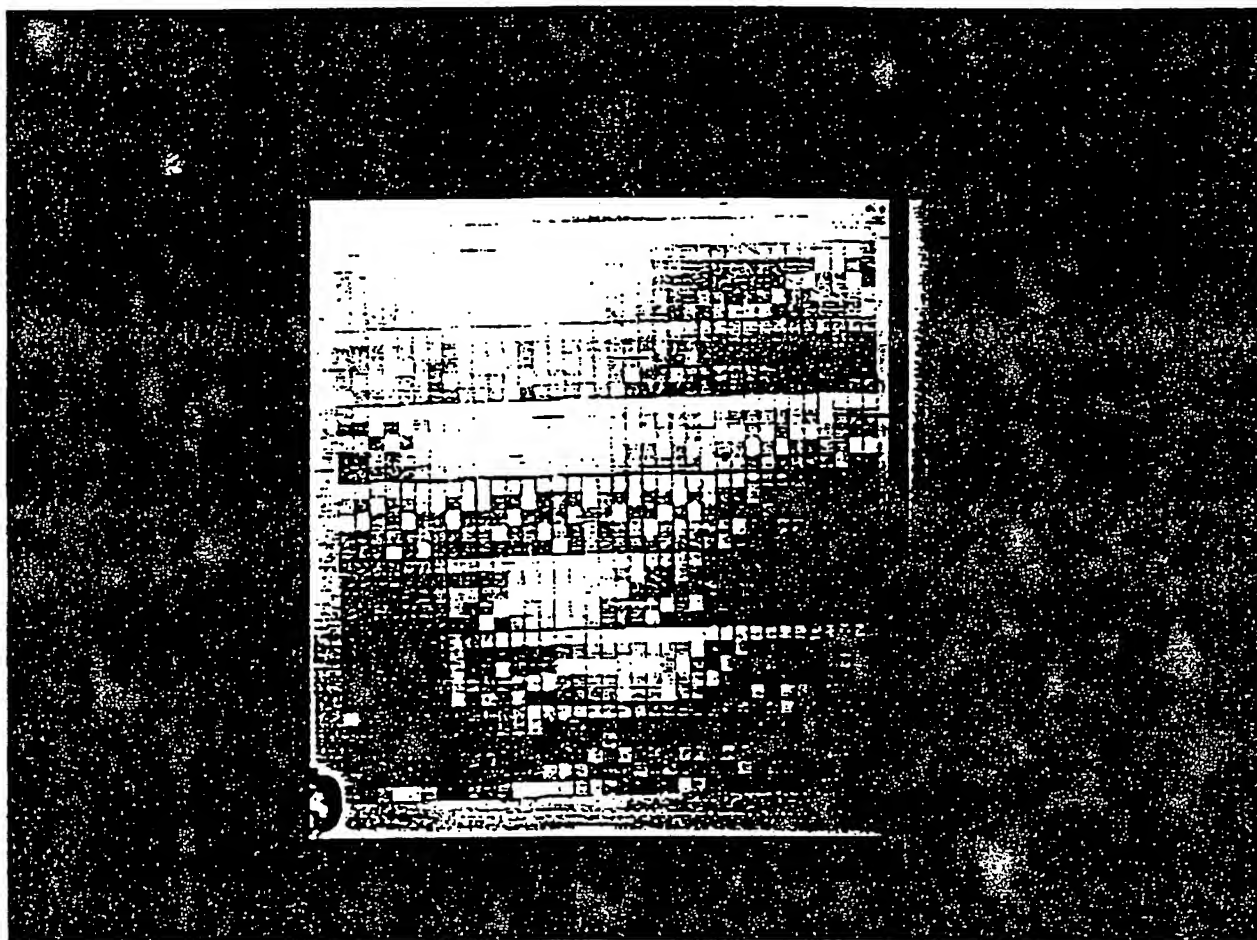


Fig. 33

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THE HUMAN MITOCHONDRIAL GENOME

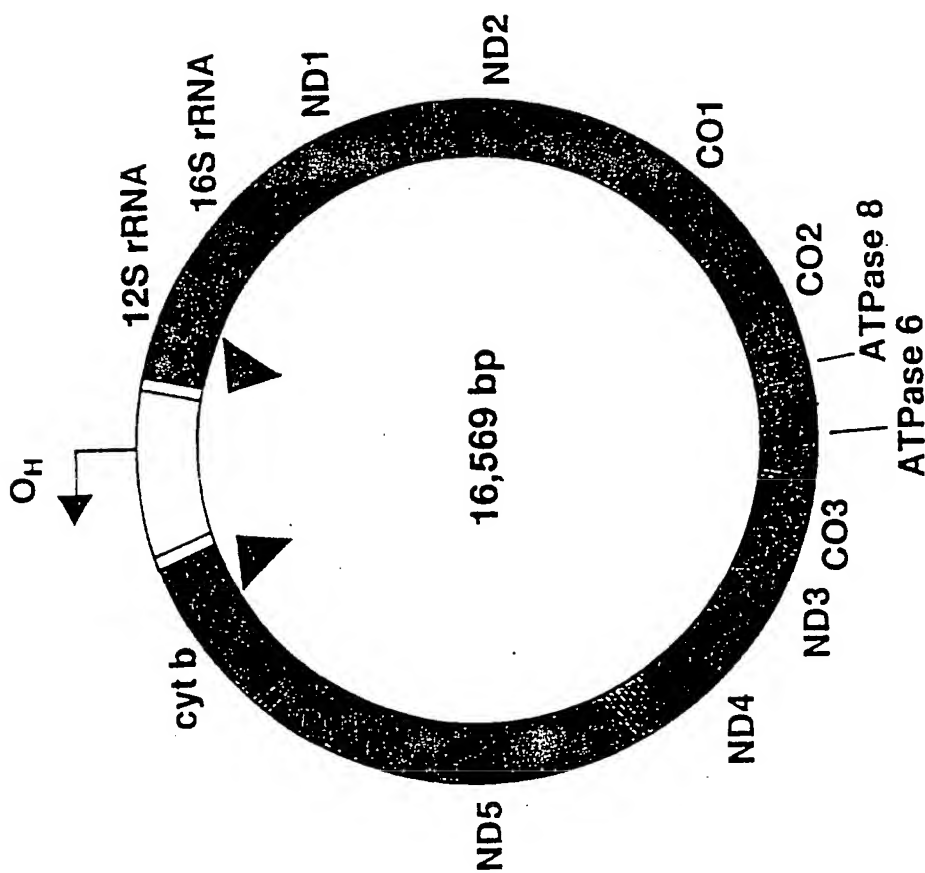
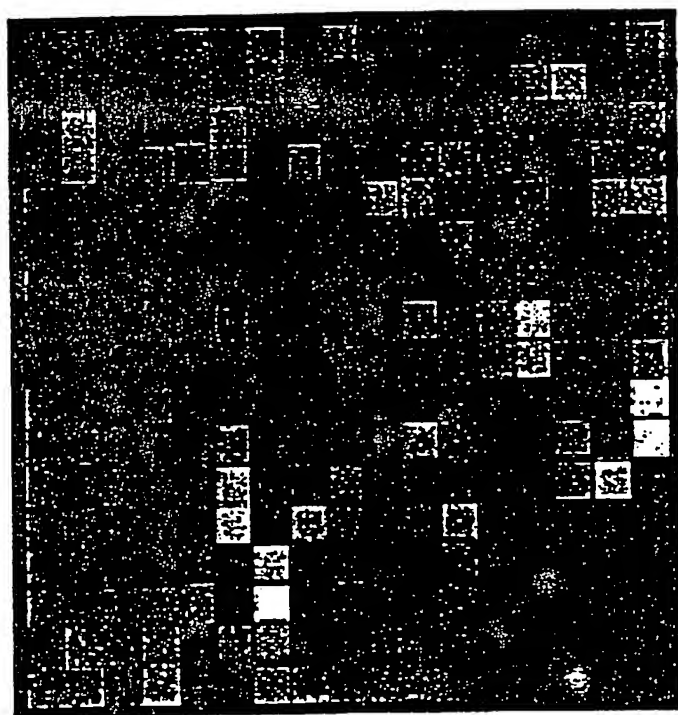


Fig. 35

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mt4

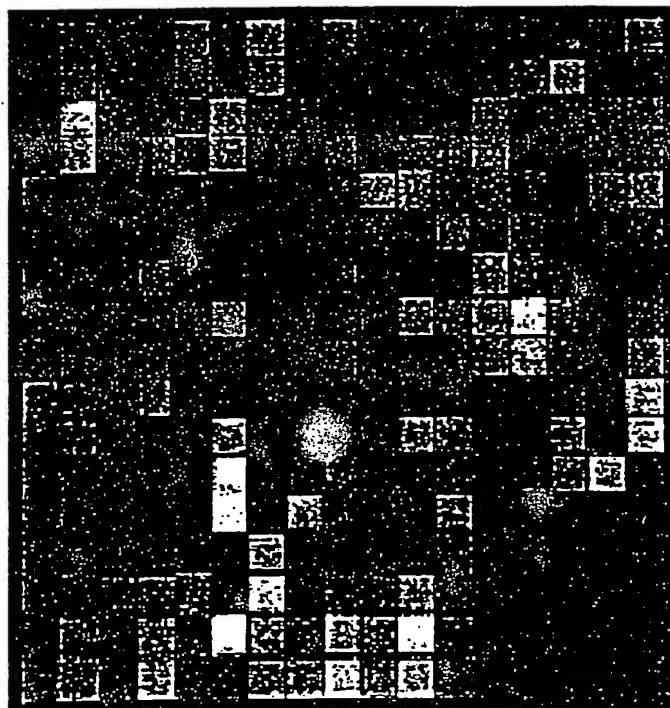


HYBRIDIZATION

Fig. 36

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HYBRIDIZATION



mt5

Fig. 37

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PREDICTED DIFFERENCE IMAGE

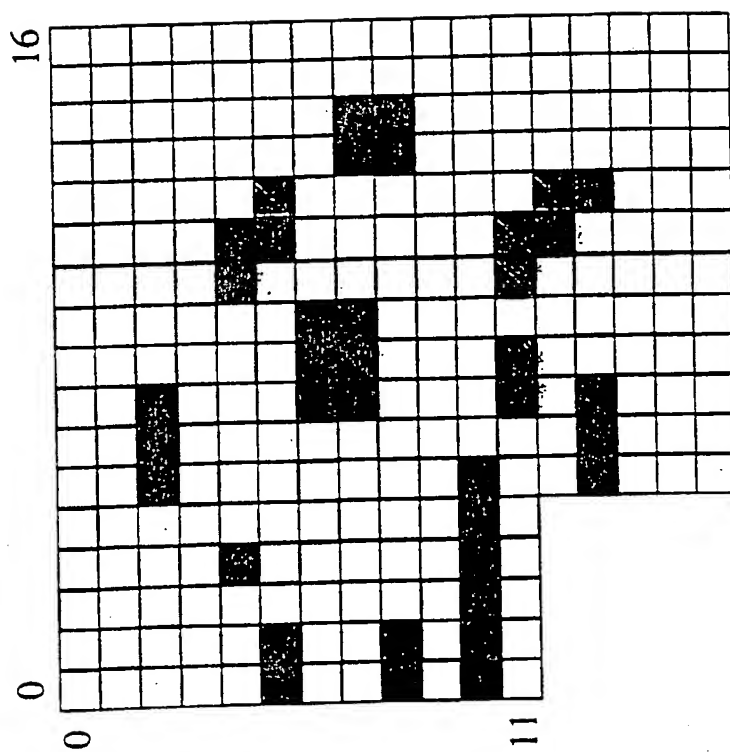


Fig. 38

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DIFFERENCE IMAGE

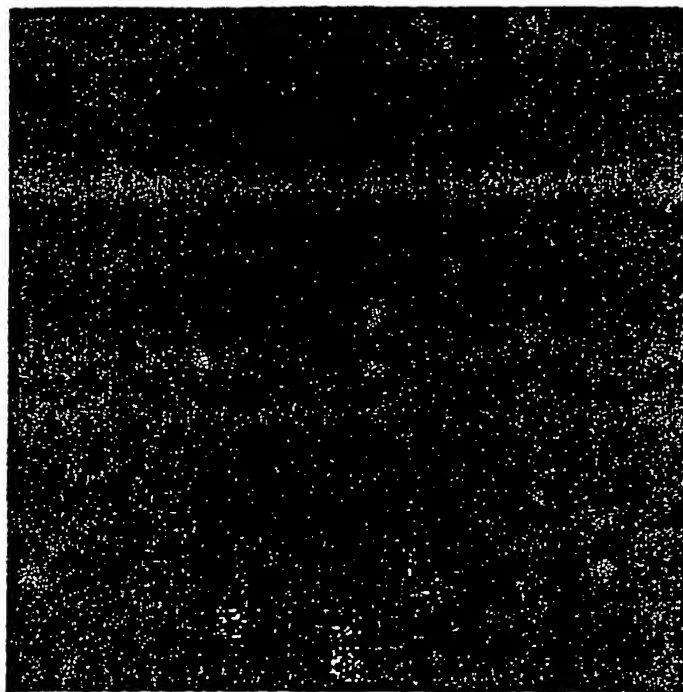


Fig. 39

NORMALIZED INTENSITIES

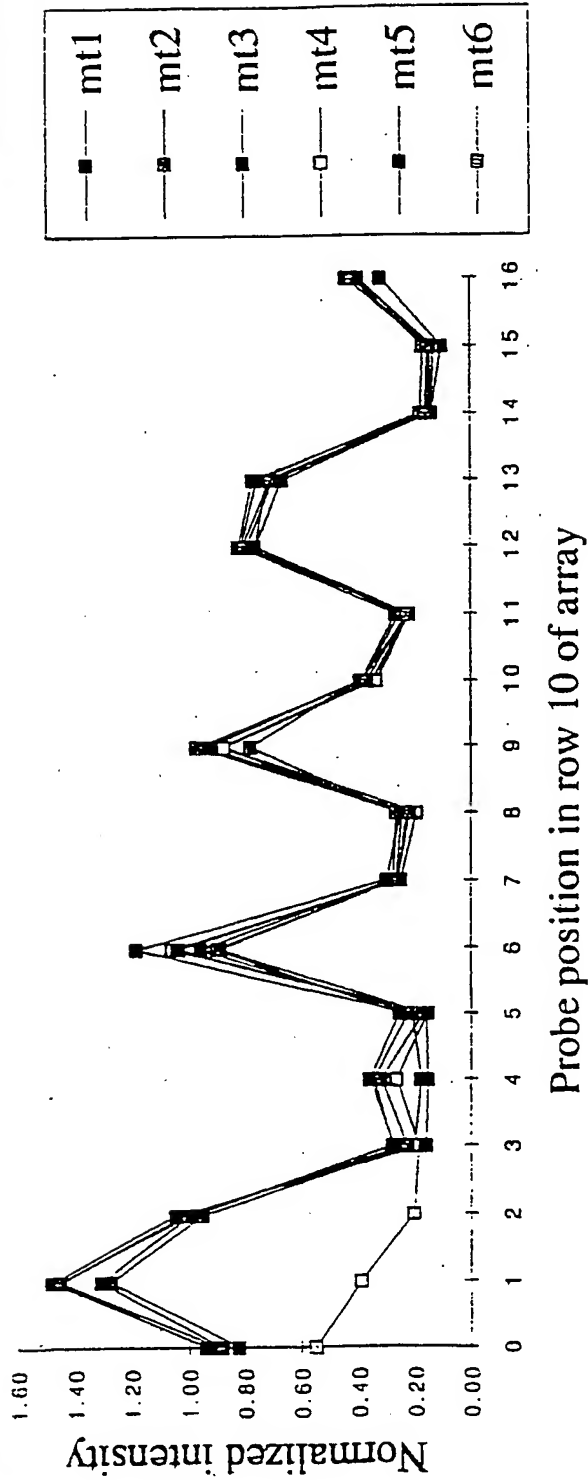


Fig. 40
Sheet 1 of 2

probe position	0	1	2	3	4	5
probe length	13	13	12	12	12	12
sample (mt1 -> 6)	4	4	4	2, 5	2, 5	2, 5
mismatch position from 3' of probe	12	5	3	12	7	2
base change	t -> a	t -> a	t -> a	t -> c	t -> c	t -> c

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NORMALIZED INTENSITIES

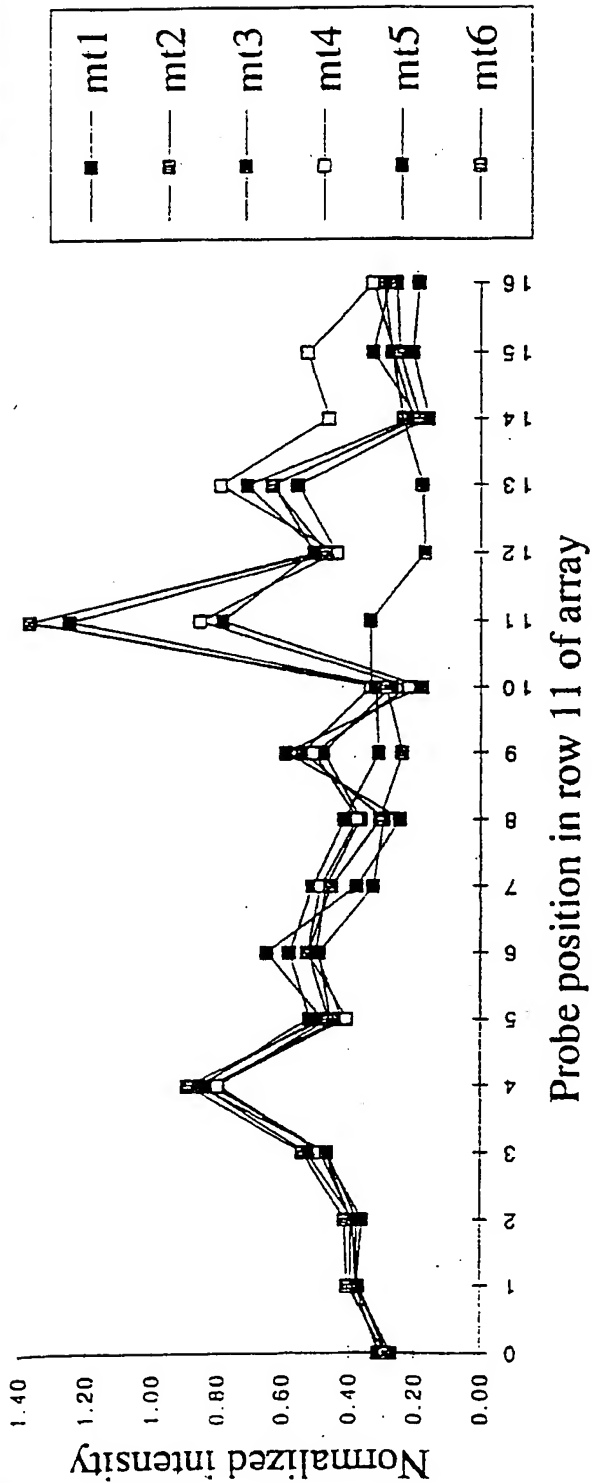


Fig. 40
Sheet 2 of 2

probe position	6	7	8	9	10	11	12	13
probe length	13	12	12	13	14	13	12	12
sample (mt1 -> 6)	2	2, 5	2, 5, 6	3, 6	3, 4, 5	2, 4, 5	2	2
mismatch position from 3' of probe	13	9, 10	3, 4 11	11, 5	4, 11, double	11, 3, double	6	3
base change	c -> t	c -> t	c -> t t -> c	t -> c	t -> c double	g -> a t -> c double	g -> a	g -> a

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DISCRIMINATION

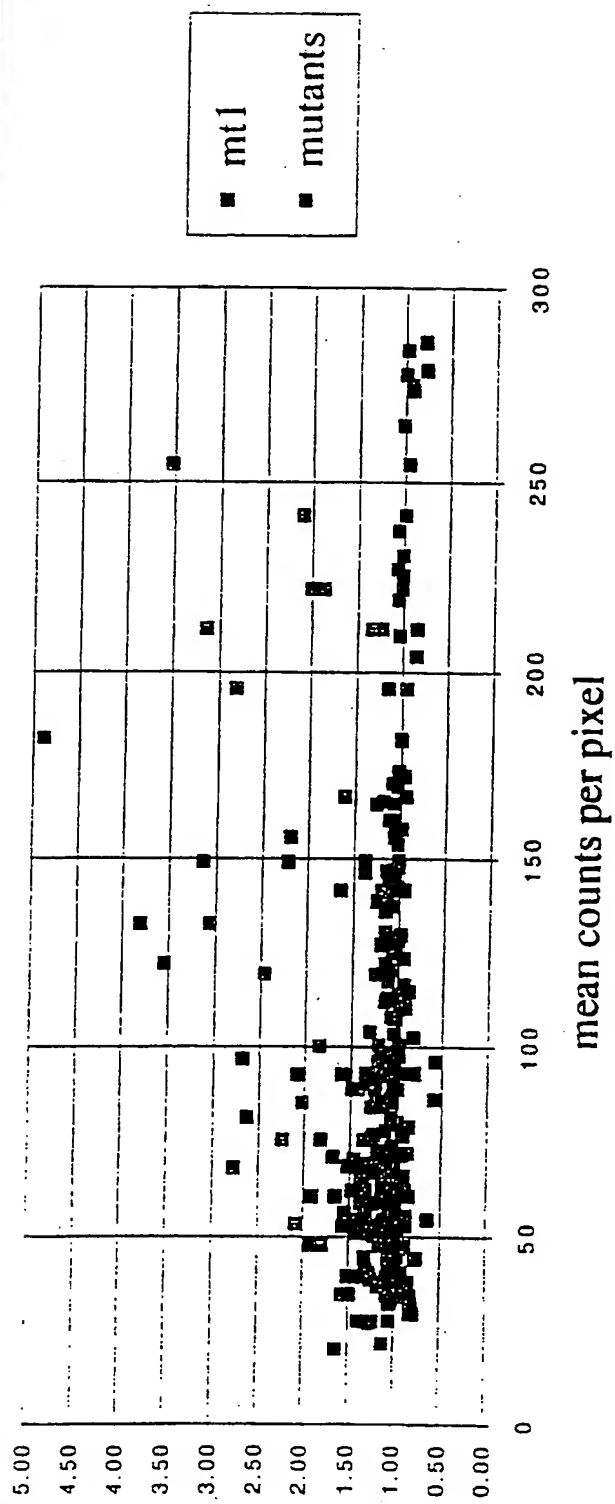


Fig. 41

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SEQUENCE & POSITION OF MUTATION

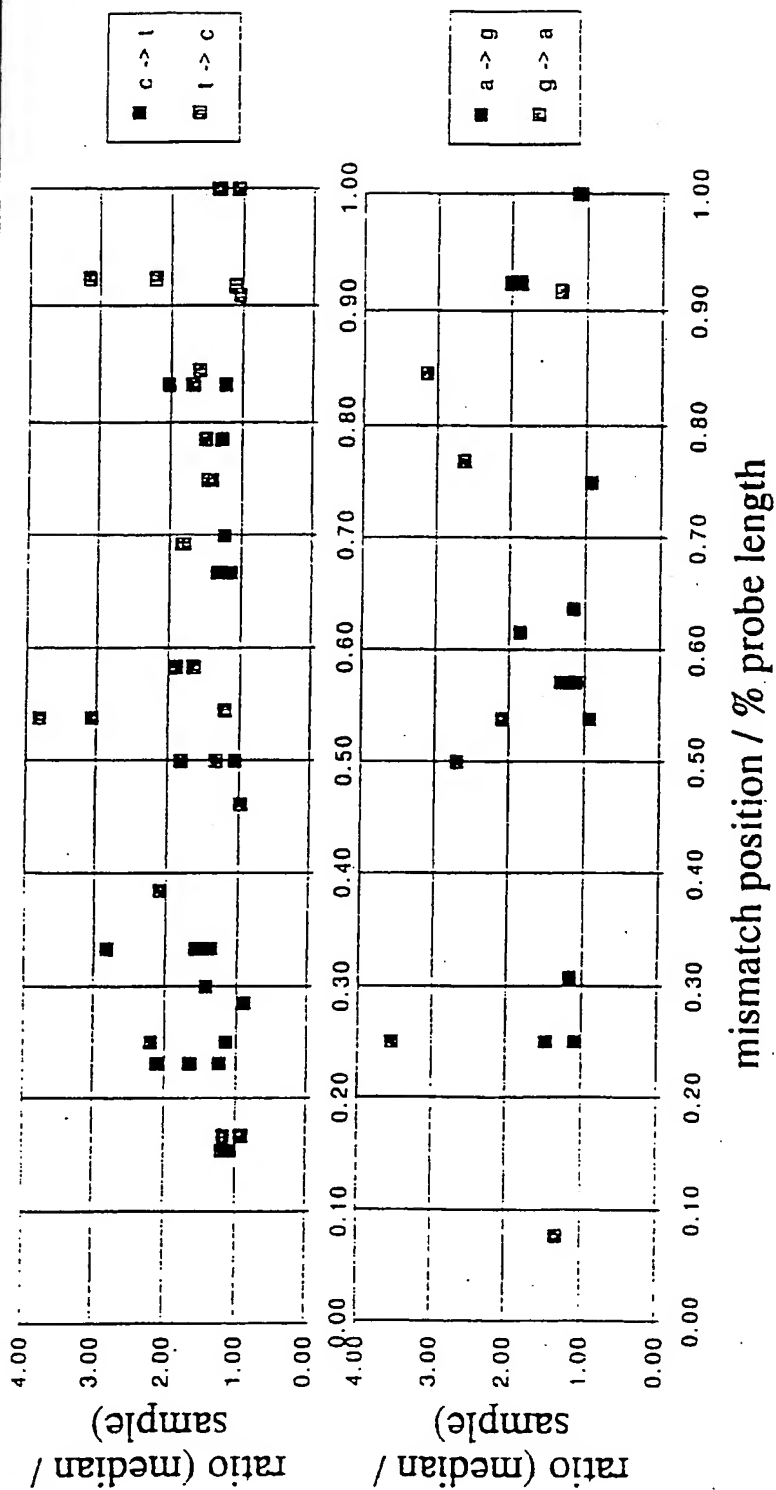


Fig. 42

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SEQUENCE

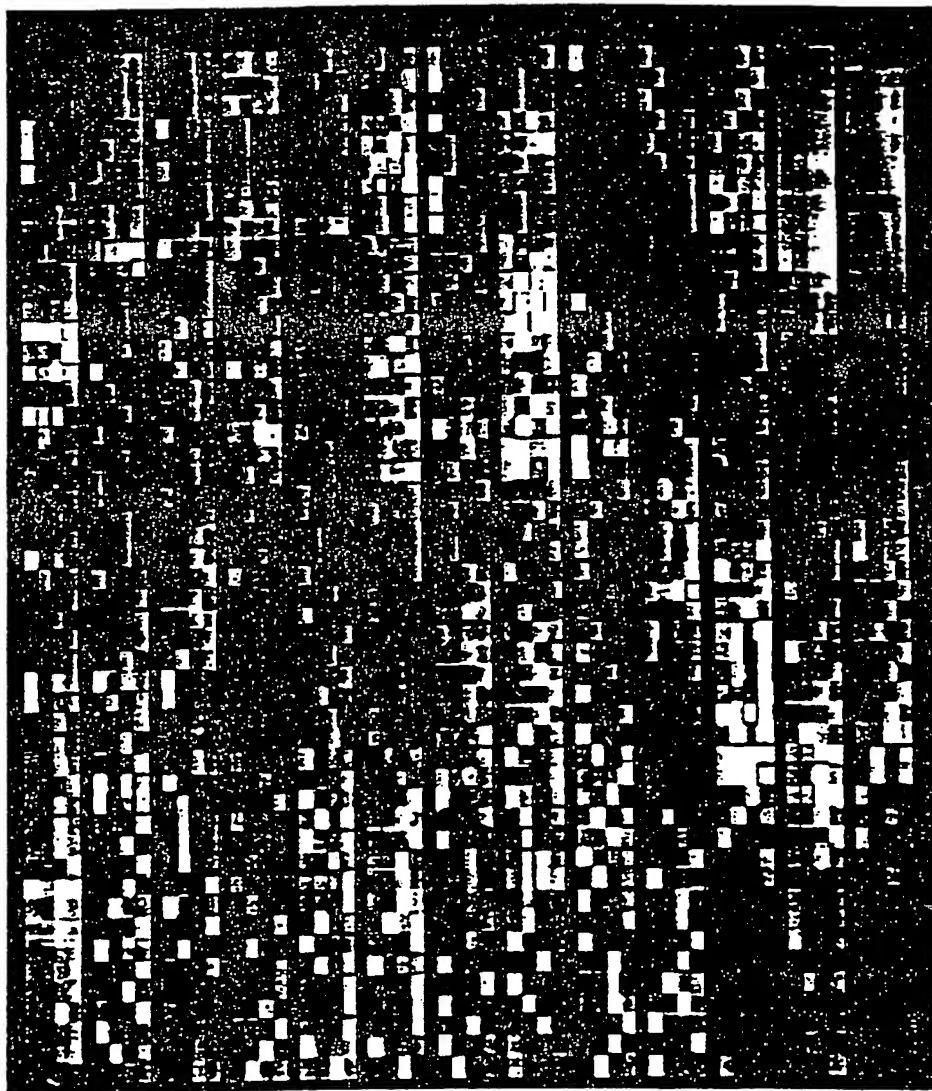
1 50
XaacaacctaccaccccttaacagtagtacataaagccatttacX
cgtacatagcacattacagtc aaatcccttctcgtcccatggatgaccc
ccctcagatagggtcccttgaccaccatccctccgtgaaatcaatatccc
gcacaagagtgcctactctcctcgctccgggcccataacacttgggggtag
ctaaagtgaactgtatccgacatctgggttcctacttcagggtTcataaagc
ctaaatagccccacacgttcccccttaaatagaacatcacgattggatcacag
gtctatcacccctattaaccactcacgggagctctccatgcatctgggtatt
ttcgtctgggggtatgcacgcgtagcatctgcgagacgctggagccgga
gcaccctatgtcgcagtatctgtctttgatctcctgcctcatcctTattatt
talcgcacctacgttcaatatctacaggcgaacatacttactaaagtgtgt
taatttaattaaatgcttgtaggacataataataacaattgaatgtctgcac
agccA ctttccacacagacatcatacaaaaaatttccaccaaaaccccc
XctcccccgcttctggccacagcacttaaacacatctTctgccaaaacccX

Fig. 43

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Fig. 44

HYBRIDIZATION



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Position:	16519	152	263	344	
Change:	T->C	T->C	A->G	T->C	
Result:					T G C A

Fig. 45

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Light Directed Oligonucleotide Synthesis

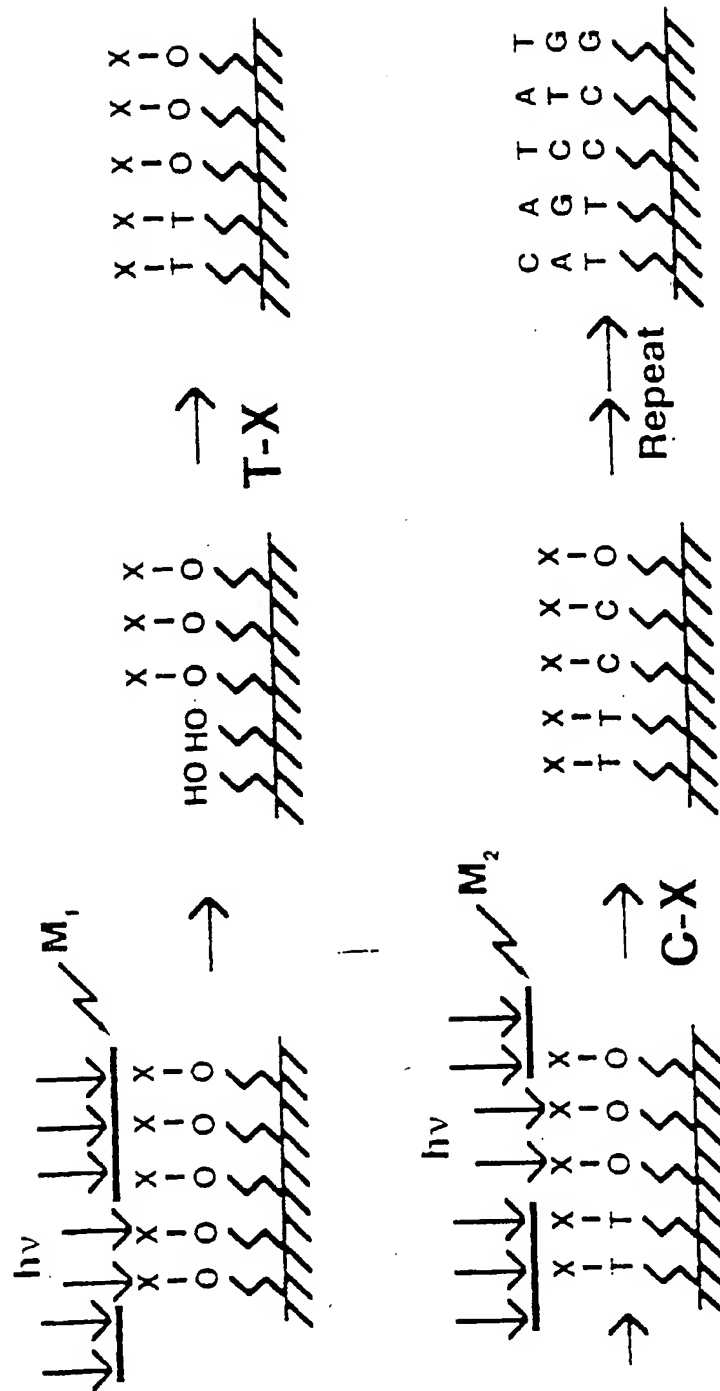
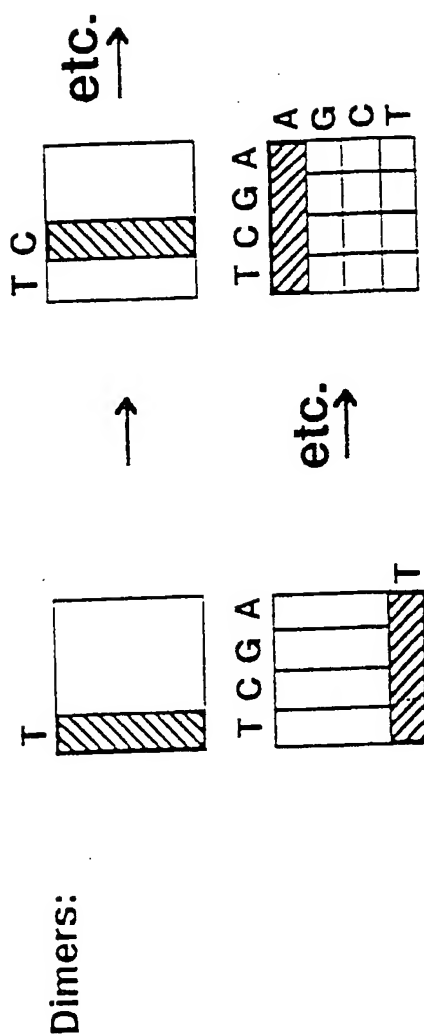


Fig. 46

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Nucleoside Combinatorials



in polynomial notation:
 $(T + C + A + G)^2 = \text{All Dimers}$

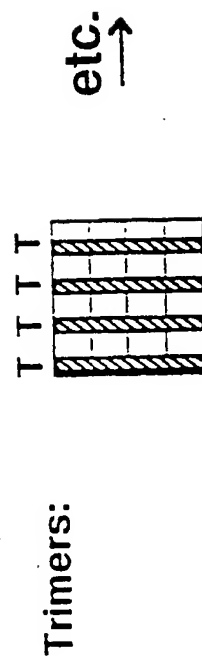
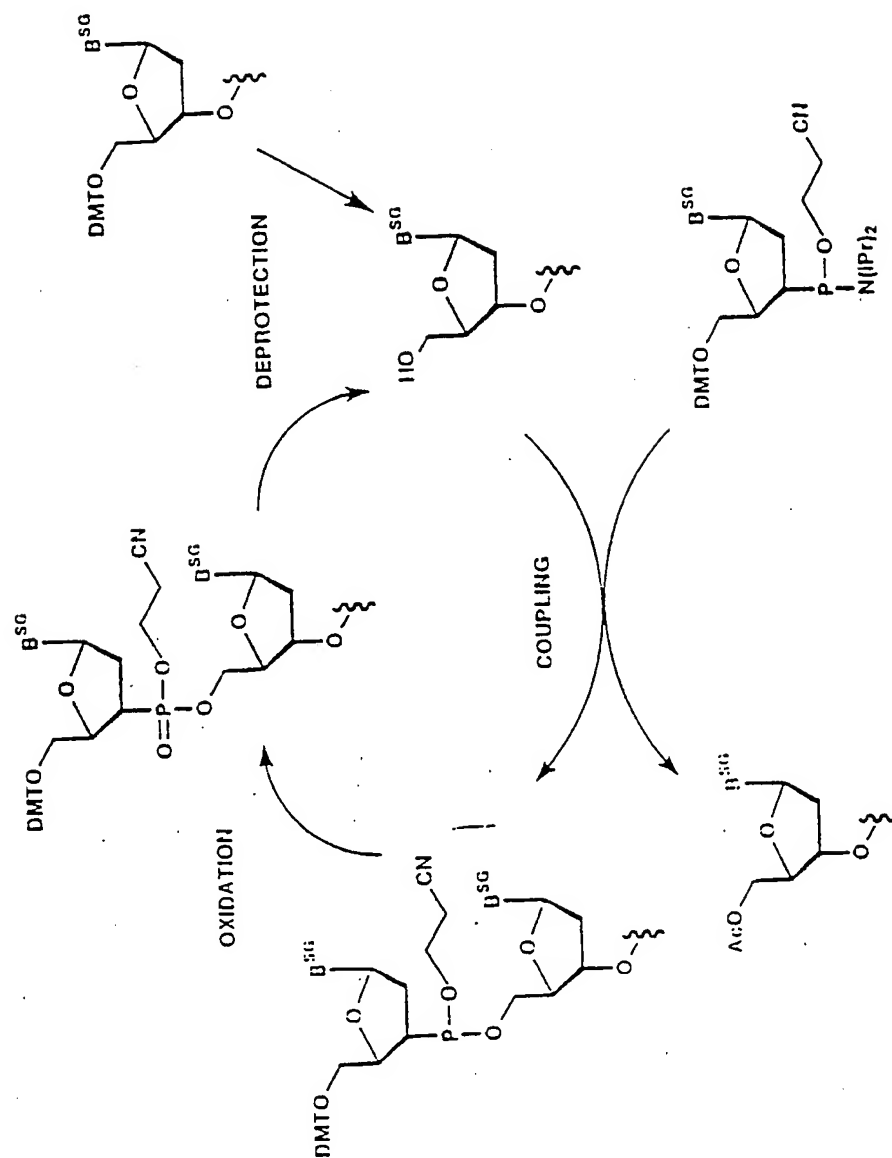


Fig. 47

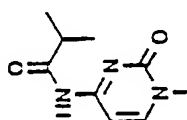
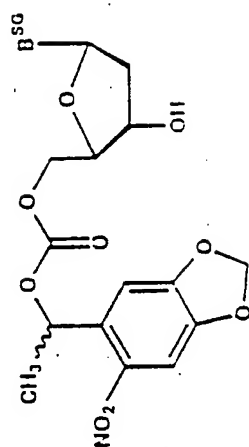
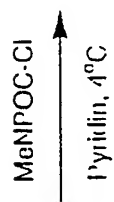
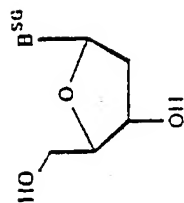
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Fig. 48

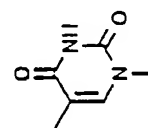
Solid Phase DNA Synthesis



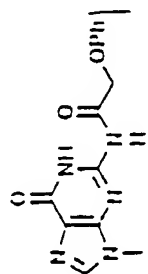
Nucleoside Buildingblocks



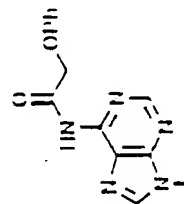
dC-IBu



dT



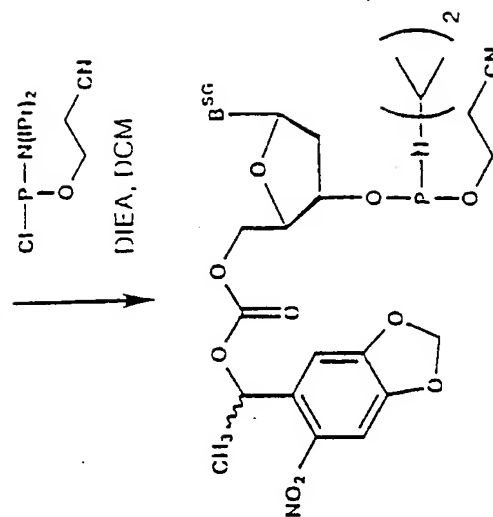
dG-Pac



dA-Pac

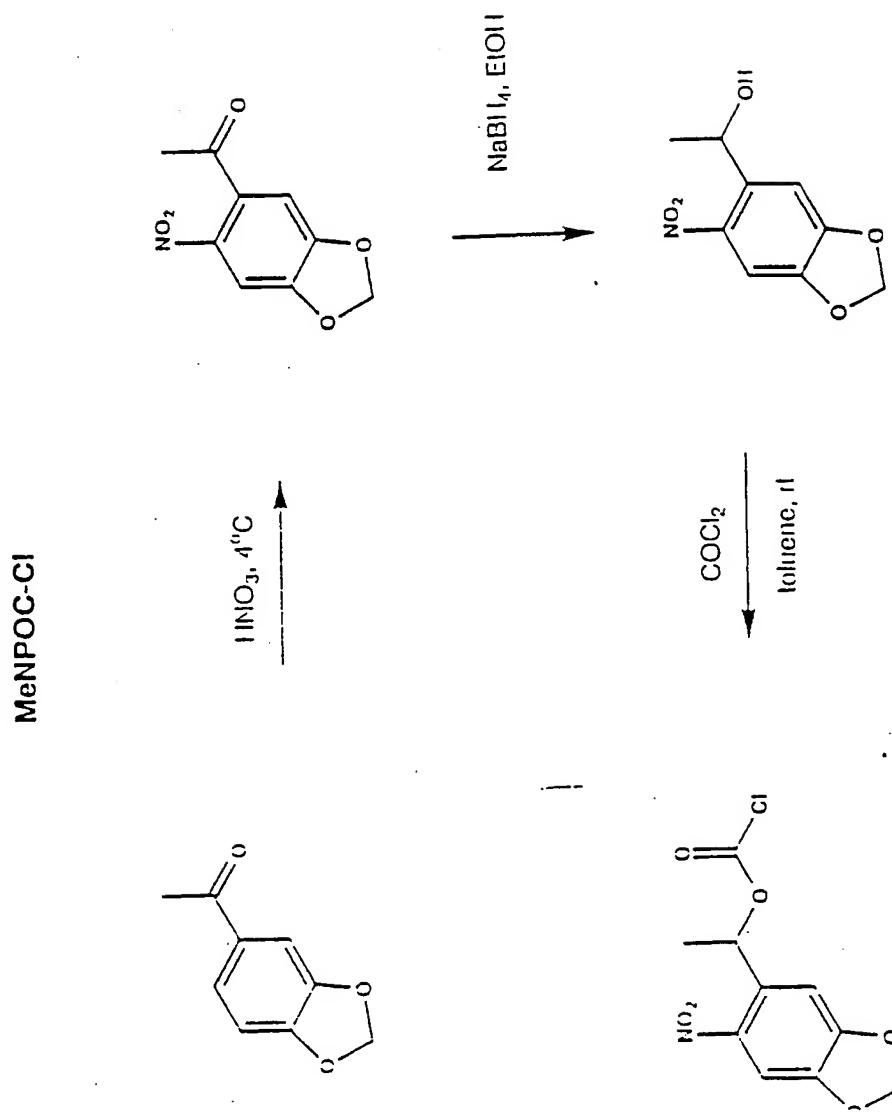
5' 3' 1'

Fig. 49



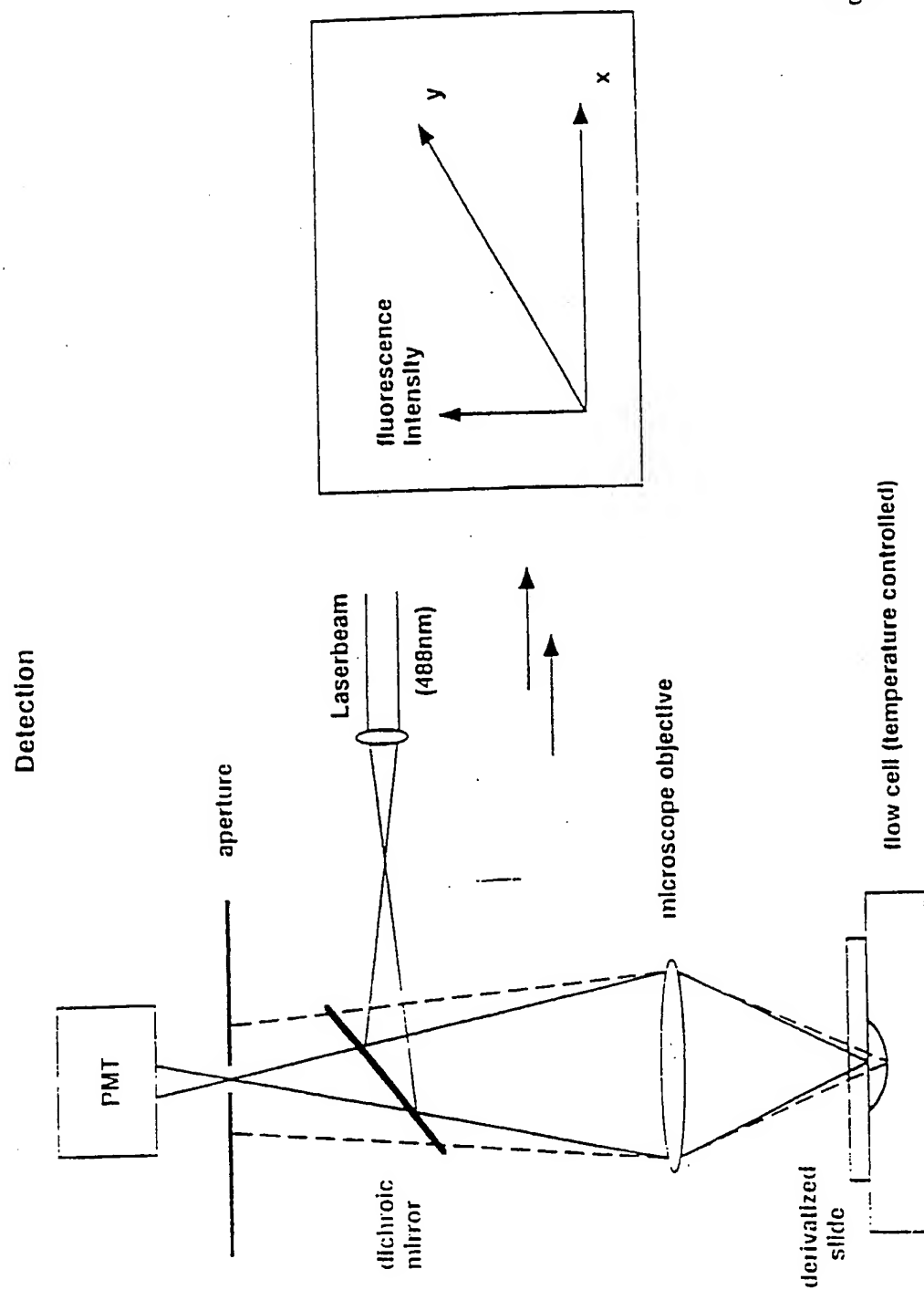
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Fig. 50



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Fig. 51



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12305

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 810; 536/22.1, 23.1, 24.3, 24.31, 24.32, 24.33, 25.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US, A, 4,656,127 (MUNDY) 07 April 1987, see especially figure 8 and example 2 in columns 10-13.	1, 7-11, 18, 28, 29, 52, 53, 60, 61, 63 ----- 2-6, 12-17, 19- 27, 30-36, 47- 51, 54-59, 62, 64-84

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* "A"	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
* "E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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* "O"	document referring to an oral disclosure, use, exhibition or other means	"Z"	document member of the same patent family
* "P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

13 FEBRUARY 1995

Date of mailing of the international search report

02 MAR 1995

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ARDIN MARSCHEL

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INTERNATIONAL SEARCH REPORT

 International application No.
 PCT/US94/12305

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X — Y	WO, A, 92/10588 (FODOR ET AL.) 25 June 1992, see the entire disclosure, especially the abstract.	1-36, 47-64 ----- 65-84
Y	Nature, Volume 313, issued 24 January 1985, Ratner et al., "Complete nucleotide sequence of the AIDS virus, HTLV-III", pages 277-284, see the entire disclosure.	65-84
Y	Virology, Volume 175, issued 1990, Querat et al., "Nucleotide Sequence Analysis of SA-OMVV, a Visna-Related Ovine Lentivirus: Phylogenetic History of Lentiviruses", pages 434-447.	64-70, 72-84
Y,P	Journal of Virology, Volume 68, Number 6, issued June 1994, Luo et al., "Cellular Protein Modulates Effects of Human Immunodeficiency Virus Type 1 Rev", pages 3850-3856, see the abstract.	64-69, 71-84
Y	Cell, Volume 40, issued January 1985, Wain-Hobson et al., "Nucleotide Sequence of the AIDS Virus, LAV", pages 9-17, see the entire disclosure.	65-84
Y	Journal of Biomolecular Structure & Dynamics, Volume 11, Number 3, issued 1993, Lipshutz, "Likelihood DNA Sequencing By Hybridization", pages 637-653, see the entire disclosure and especially the abstract.	1-36, 47-84
Y	Maximum Entropy and Bayesian Methods (Paris), issued 1992, Elder, "Analysis of DNA Oligonucleotide Hybridization Data by Maximum Entropy", pages 1-10, see especially the abstract and the discussion relating to Figure 2 on page 6.	1-36, 47-84
Y	WO, A, 89/10977 (SOUTHERN) 16 November 1989, see especially the abstract and claims 1-14.	1-36, 47-84
X — Y	Genomics, Volume 13, issued 1992, Southern et al., "Analyzing and Comparing Nucleic Acid Sequences by Hybridization to Arrays of Oligonucleotides: Evaluation Using Experimental Models", pages 1008-1017, see especially the abstract and Figures 2-4 on pages 1010-1012.	1-36, 47-64 ----- 65-84
X — Y	WO, A, 93/17126 (CHETVERIN ET AL.) 02 September 1993, see especially the abstract and claims 1-197.	1-36, 47-64 ----- 65-84

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12305

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US, A, 5,002,867 (MACEVICZ) 26 March 1991, see especially the abstract and claims 1-23.	1-36, 47-64 ----- 65-84
X --- Y	US, A, 5,202,231 (DRMANAC ET AL.) 13 April 1993, see especially the abstract and claims 1-4.	1-36, 47-64 ----- 65-84

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12305

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
1-36 and 47-84

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/12305

A. CLASSIFICATION OF SUBJECT MATTER: IPC (6):

C12Q 1/68; C07H 21/02, 21/04

A. CLASSIFICATION OF SUBJECT MATTER: US CL :

435/6; 536/22.1, 23.1, 24.3, 24.31, 24.32

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

CAS, BIOSIS, WORLD PATENT INDEX, BIOTECH ABS., MEDLINE
search terms: probes, arrays, hybridization, matrix, sequencing, probe set

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Group I, claims 1-36 and 47-84, drawn to arrays of oligonucleotide probes including specifically HIV directed arrays and methods of using said arrays via hybridization to target nucleic acid.

Group II, claims 37-41, drawn to methods of using arrays of pools of probes for the comparison of a target sequence with a reference sequence.

Group III, claims 42-46, drawn to pooled probes and arrays of pooled probes immobilized on a solid support.

Group IV, claims 85-96, drawn to arrays directed to reference sequences directed to the CFTR gene.

Group V, claims 97, 98, and 100-103, drawn to arrays directed to reference sequences directed to the p53 and hMLH1 genes.

Group VI, claim 99, drawn to arrays directed to reference sequences directed to the MSH2 gene.

Group VII, claims 104-108, drawn to arrays directed to reference sequences directed to sequences from the mitochondrial genome.

The inventions listed as Groups I-VII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

The arrays and methods of use in the invention of Group I utilize probe sets having interrogation positions wherein where hybridization of the target nucleic acid to certain probes results in determining whether the target nucleic acid is the same or different from the reference sequence. Certain claims are directed specifically to HIV reference sequences. The special technical feature is deemed to be the practice of probe sets wherein specific hybridization to certain probes produces an indication of whether the interrogation position is the same or different from the reference sequence wherein the target sequence is determined by the compilation of interrogation sequence results to obtain the entire sequence. The first claimed specific reference sequence is directed to HIV. In contrast, Groups II and III cite the practice of pooled probes with variant sequences therein which are exactly complementary to each variant target sequence. The intensity of hybridization to each pool is the manner of determining the comparison between the target nucleic acid and the reference sequence. Groups II and III therefore do not determine the target sequence using the special technical features cited above but instead signal intensity using pooled probes. Therefore unity of invention is lacking between Group I and Groups II and III. Groups II and III also lack unity of invention with each other because Group II is directed to methods of using Group II is directed to the use of arrays of pooled probes whereas Group III is

INTERNATIONAL SEARCH REPORT

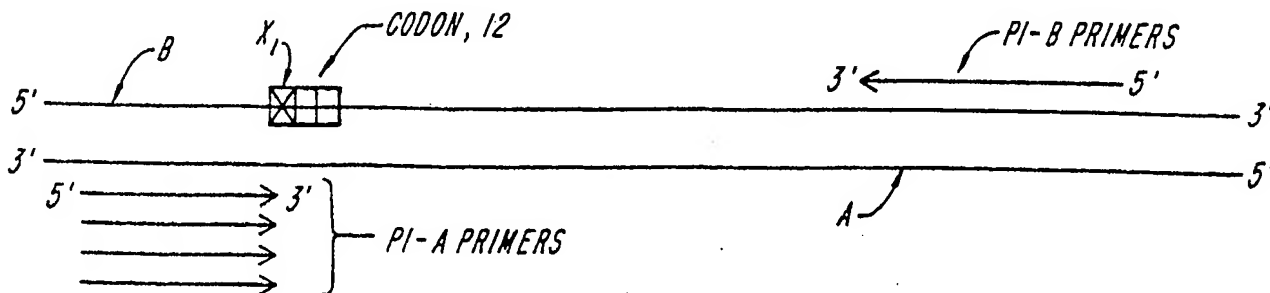
International application No.

PCT/US94/12305

directed only to a pool of probes which may have many other uses and also contain limitations therein to specific positions in probes in the pool which are not recited in Group II. Thus, Groups II and III lack unity of invention in not containing the same special technical feature for probes in a pool or pools therein. Groups IV-VII all are directed to arrays similar to that cited in Group I but are directed to completely different specific reference genes. Therefore Groups IV-VII lack unity of invention with Groups II and III for the same reasons as discussed above regarding Group I. Additionally the completely different and totally unrelated specific reference genes cited in Groups IV-VII therefore are directed to a different specific reference gene which is deemed the special technical feature of these Groups when each of Groups I and IV-VII are compared to any other Group therein. In summary the claims are not so linked by a special technical feature within the meaning of PCT Rule 13.2 so as to form a single inventive concept.

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12Q 1/68, C12P 19/34 C07H 21/04	A1	(11) International Publication Number: WO 93/22456 (43) International Publication Date: 11 November 1993 (11.11.93)
(21) International Application Number: PCT/US93/03561 (22) International Filing Date: 14 April 1993 (14.04.93) (30) Priority data: 07/874,845 27 April 1992 (27.04.92) US (71) Applicant: TRUSTEES OF DARTMOUTH COLLEGE [US/US]; 309 McNutt Building, P.O. Box 7, Hanover, NH 03755 (US). (72) Inventor: SORENSON, George, D. ; P.O. Box 176, Merid- en, NH 03770 (US). (74) Agents: GEARY, William, C., III et al.; Lahive & Cock- field, 60 State Street, Boston, MA 02109 (US).		(81) Designated States: CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>

(54) Title: DETECTION OF GENE SEQUENCES IN BIOLOGICAL FLUIDS**(57) Abstract**

Methods are provided for detecting and quantitating gene sequences, such as mutated genes and oncogenes, in biological fluids. The fluid sample (e.g., plasma, serum, urine, etc.) is obtained, deproteinized and the DNA present in the sample is extracted. Following denaturation of the DNA, an amplification procedure, such as PCR or LCR, is conducted to amplify the mutated gene sequence.

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ES	Spain	MN	Mongolia	VN	Viet Nam
FI	Finland				

DETECTION OF GENE SEQUENCES IN BIOLOGICAL FLUIDS

Government Support

The research leading to this invention was supported by government funding pursuant to NIH Grant No. CA 47248.

Background of the Invention

Soluble DNA is known to exist in the blood of healthy individuals at concentrations of about 5 to 10 ng/ml. It is believed that soluble DNA is present in increased levels in the blood of individuals having autoimmune diseases, particularly systemic lupus erythematosus (SLE) and other diseases including viral hepatitis, cancer and pulmonary embolism. It is not known whether circulating soluble DNA represents a specific type of DNA which is particularly prone to appear in the blood. However, studies indicate that the DNA behaves as double-stranded DNA or as a mixture of double-stranded and single-stranded DNA, and that it is likely to be composed of native DNA with single-stranded regions. Dennin, R.H., Klin. Wochenschr. 57:451-456, (1979). Steinman, C.R., J. Clin. Invest., 73:832-841, (1984). Fournie, G.J. et al., Analytical Biochem. 158:250-256, (1986). There is also evidence that in patients with SLE, the circulating DNA is enriched for human repetitive sequence (Alu) containing fragments when compared to normal human genomic DNA.

In patients with cancer, the levels of circulating soluble DNA in blood are significantly increased. Types of cancers which appear to have a high incidence of elevated DNA levels include pancreatic carcinoma, breast carcinoma, colorectal carcinoma and pulmonary carcinoma. In these forms of cancer, the levels of circulating soluble DNA in blood are usually over 50 ng/ml, and generally the mean values are more than 150 ng/ml. Leon et al., Can. Res. 37:646-650, 1977; Shapiro et al., Cancer 51:2116-2120, 1983.

Mutated oncogenes have been described in experimental and human tumors. In some instances certain mutated oncogenes are associated with particular types of tumors. Examples of these are adenocarcinomas of the pancreas, colon and lung which have approximately a 75%, 50%, and 35% incidence respectively, of Kirsten ras (K-ras) genes with mutations in positions 1 or 2 of codons 12. The most frequent mutations are changes from glycine to valine (GGT to GTT), glycine to cysteine (GGT to TGT), and glycine to aspartic acid (GGT to GAT). Other, but less common mutations of codon 12 include mutations to AGT and CGT. K-ras genes in somatic cells of such patients are not mutated.

The ability to detect sequences of mutated oncogenes or other genes in small samples of biological fluid, such as blood plasma, would provide a useful diagnostic tool. The presence of mutated K-ras gene sequences in the plasma would be indicative of the presence in the patient of a tumor

which contains mutated oncogenes. Presumably this would be a specific tumor marker since there is no other known source of mutated K-ras genes. Therefore, this evaluation may be useful in suggesting and/or confirming a diagnosis. The amount of mutated K-ras sequences in the plasma may relate to the size of the tumor, the growth rate of the tumor and/or the regression of the tumor. Therefore, serial quantitation of mutated K-ras sequences may be useful in determining changes in tumor mass. Since most human cancers have mutated oncogenes, evaluation of plasma DNA for mutated sequences may have very wide applicability and usefulness.

Summary Of The Invention

This invention recognizes that gene sequences (e.g., oncogene sequences) exist in blood, and provides a method for detecting and quantitating gene sequences such as from mutated oncogenes and other genes in biological fluids, such as blood plasma and serum. The method can be used as a diagnostic technique to detect certain cancers and other diseases which tend to increase levels of circulating soluble DNA in blood. Moreover, this method is useful in assessing the progress of treatment regimes for patients with certain cancers.

The method of the invention involves the initial steps of obtaining a sample of biological fluid (e.g., urine, blood plasma or serum, sputum, cerebral spinal fluid), then deproteinizing and extracting the DNA. The DNA is then amplified by

techniques such as the polymerase chain reaction (PCR) or the ligase chain reaction (LCR) in an allele specific manner to distinguish a normal gene sequence from a mutated gene sequence present in the sample. In one embodiment where the location of the mutation is known, the allele specific PCR amplification is performed using four pairs of oligonucleotide primers. The four primer pairs include a set of four allele specific first primers complementary to the gene sequence contiguous with the site of the mutation on the first strand. These four primers are unique with respect to each other and differ only at the 3' nucleotide which is complementary to the wild type nucleotide or to one of the three possible mutations which can occur at this known position. The four primer pairs also include a single common primer which is used in combination with each of the four unique first strand primers. The common primer is complementary to a segment of a second strand of the DNA, at some distance from the position of the first primer.

This amplification procedure amplifies a known base pair fragment which includes the mutation. Accordingly, this technique has the advantage of displaying a high level of sensitivity since it is able to detect only a few mutated DNA sequences in a background of a 10^7 -fold excess of normal DNA. The method is believed to be of much greater sensitivity than methods which detect point mutations by hybridization of a PCR product with allele specific radiolabelled probes which will not detect a mutation if the normal DNA is in more than 20-fold excess.

The above embodiment is useful where a mutation exists at a known location on the DNA. In another embodiment where the mutation is known to exist in one of two possible positions, eight pair of oligonucleotide primers may be used. The first set of four primer pairs (i.e., the four unique, allele specific primers, each of which forms a pair with a common primer) is as described above. The second set of four primer pairs comprises four allele specific primers complementary to the gene sequence contiguous with the site of the second possible mutation on the sense strand. These four primers are unique with respect to each other and differ at the terminal 3' nucleotide which is complementary to the wild type nucleotide or to one of the three possible mutations which can occur at this second known position. Each of these allele specific primers is paired with another common primer complementary to the other strand, distant from the location of the mutation.

The PCR techniques described above preferably utilize a DNA polymerase which lacks 3' exonuclease activity and therefore the ability to proofread. A preferred DNA polymerase is Thermus aquaticus DNA polymerase.

During the amplification procedure, it is usually sufficient to conduct approximately 30 cycles of amplification in a DNA thermal cycler. After an initial denaturation period of 5 minutes, each amplification cycle preferably includes a denaturation period of about 1 minute at 95°C., primer annealing for about 2 minutes at 58°C and an extension at 72°C for approximately 1 minute.

Following the amplification, aliquots of amplified DNA from the PCR can be analyzed by techniques such as electrophoresis through agarose gel using ethidium bromide staining. Improved sensitivity may be attained by using labelled primers and subsequently identifying the amplified product by detecting radioactivity or chemiluminescence on film. Labelled primers may also permit quantitation of the amplified product which may be used to determine the amount of target sequence in the original specimen.

As used herein, allele specific amplification describes a feature of the method of the invention where primers are used which are specific to a mutant allele, thus enabling amplification of the sequence to occur where there is 100% complementarity between the 3' end of the primer and the target gene sequence. Thus, allele specific amplification is advantageous in that it does not permit amplification unless there is a mutated allele. This provides an extremely sensitive detection technique.

Brief Description Of The Drawings

Figures 1A and 1B are diagrammatic representations of the amplification strategy for the detection of a mutated K-ras gene with a mutation present at a single known location of K-ras.

Figures 2A and 2B are diagrammatic representations of the amplification strategy for detection of a mutated K-ras gene with a mutation present at a second of two possible locations of K-ras.

Detailed Description of The Invention

The detection of mutated DNA, such as specific single copy genes, is potentially useful for diagnostic purposes, and/or for evaluating the extent of a disease. Normal plasma is believed to contain about 10 ng of soluble DNA per ml. The concentration of soluble DNA in blood plasma is known to increase markedly in individuals with cancer and some other diseases. The ability to detect the presence of known mutated gene sequences, such as K-ras gene sequences, which are indicative of a medical condition, is thus highly desirable.

The present invention provides a highly sensitive diagnostic method enabling the detection of such mutant alleles in biological fluid, even against a background of as much as a 10^7 -fold excess of normal DNA. The method generally involves the steps of obtaining a sample of a biological fluid containing soluble DNA, deproteinizing, extracting and denaturing the DNA, followed by amplifying the DNA in an allele specific manner, using a set of primers among which is a primer specific for the mutated allele. Through this allele specific amplification technique, only the mutant allele is amplified. Following amplification, various

techniques may be employed to detect the presence of amplified DNA and to quantify the amplified DNA. The presence of the amplified DNA represents the presence of the mutated gene, and the amount of the amplified gene present can provide an indication of the extent of a disease.

This technique is applicable to the identification in biological fluid of sequences from single copy genes, mutated at a known position on the gene. Samples of biological fluid having soluble DNA (e.g., blood plasma, serum, urine, sputum, cerebral spinal fluid) are collected and treated to deproteinize and extract the DNA. Thereafter, the DNA is denatured. The DNA is then amplified in an allele specific manner so as to amplify the gene bearing a mutation.

During deproteinization of DNA from the fluid sample, the rapid removal of protein and the virtual simultaneous deactivation of any DNase is believed to be important. DNA is deproteinized by adding to aliquots of the sample an equal volume of 20% NaCl and then boiling the mixture for about 3 to 4 minutes. Subsequently, standard techniques can be used to complete the extraction and isolation of the DNA. A preferred extraction process involves concentrating the amount of DNA in the fluid sample by techniques such as centrifugation.

The use of the 20% NaCl solution, followed by boiling, is believed to rapidly remove protein and simultaneously inactivate any DNases present. DNA

present in the plasma is believed to be in the form of nucleosomes and is thus believed to be protected from the DNases while in blood. However, once the DNA is extracted, it is susceptible to the DNases. Thus, it is important to inactivate the DNases at the same time as deproteinization to prevent the DNases from inhibiting the amplification process by reducing the amount of DNA available for amplification. Although the 20% NaCl solution is currently preferred, it is understood that other concentrations of NaCl, and other salts, may also be used.

Other techniques may also be used to extract the DNA while preventing the DNases from affecting the available DNA. Because plasma DNA is believed to be in the form of nucleosomes (mainly histones and DNA), plasma DNA could also be isolated using an antibody to histones or other nucleosomal proteins. Another approach could be to pass the plasma (or serum) over a solid support with attached antihistone antibodies which would bind with the nucleosomes. After rinsing the nucleosomes can be eluted from the antibodies as an enriched or purified fraction. Subsequently, DNA can be extracted using the above or other conventional methods.

In one embodiment, the allele specific amplification is performed through the Polymerase Chain Reaction (PCR) using primers having 3' terminal nucleotides complementary to specific point mutations of a gene for which detection is sought. PCR preferably is conducted by the method described by Saiki, "Amplification of Genomic DNA", PCR Protocols,

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Eds. M.A. Innis, et al., Academic Press, San Diego (1990), pp. 13. In addition, the PCR is conducted using a thermostable DNA polymerase which lacks 3' exonuclease activity and therefore the ability to repair single base mismatches at the 3' terminal nucleotide of the DNA primer during amplification. As noted, a preferred DNA polymerase is T. aquaticus DNA polymerase. A suitable T. aquaticus DNA polymerase is commercially available from Perkin-Elmer as AmpliTaq DNA polymerase. Other useful DNA polymerases which lack 3' exonuclease activity include a Ventr (exo-), available from New England Biolabs, Inc., (purified from strains of E. coli that carry a DNA polymerase gene from the archaeobacterium Thermococcus litoralis), Hot Tub DNA polymerase derived from Thermus flavus and available from Amersham Corporation, and Tth DNA polymerase derived from Thermus thermophilus, available from Epicentre Technologies, Molecular Biology Resource Inc., or Perkin-Elmer Corp.

This method conducts the amplification using four pairs of oligonucleotide primers. A first set of four primers comprises four allele specific primers which are unique with respect to each other. The four allele specific primers are each paired with a common distant primer which anneals to the other DNA strand distant from the allele specific primer. One of the allele specific primers is complementary to the wild type allele (i.e., is allele specific to the normal allele) while the others have a mismatch at the 3' terminal nucleotide of the primer. As noted, the four unique primers are individually paired for

amplification (e.g., by PCR amplification) with a common distant primer. When the mutated allele is present, the primer pair including the allele specific primer will amplify efficiently and yield a detectable product. While the mismatched primers may anneal, the strand will not be extended during amplification.

The above primer combination is useful where a mutation is known to exist at a single position on an allele of interest. Where the mutation may exist at one of two locations, eight pair of oligonucleotide primers may be used. The first set of four pair is as described above. The second four pair or primers comprises four allele specific oligonucleotide primers complementary to the gene sequence contiguous with the site of the second possible mutation on the sense strand. These four primers differ at the terminal 3' nucleotide which is complementary to the wild type nucleotide or to one of the three possible mutations which can occur at this second known position. Each of the four allele specific primers is paired with a single common distant primer which is complementary to the antisense strand upstream of the mutation.

During a PCR amplification using the above primers, only the primer which is fully complementary to the allele which is present will anneal and extend. The primers having a non-complementary nucleotide may partially anneal, but will not extend during the amplification process. Amplification generally is allowed to proceed for a suitable number

of cycles, i.e., from about 20 to 40, and most preferably for about 30. This technique amplifies a mutation-containing fragment of the target gene with sufficient sensitivity to enable detection of the mutated target gene against a significant background of normal DNA.

The K-ras gene has point mutations which usually occur at one or two known positions in a known codon. Other oncogenes may have mutations at known but variable locations. Mutations with the K-ras gene are typically known to be associated with certain cancers such as adenocarcinomas of the lung, pancreas, and colon. Figures 1A through 2B illustrate a strategy for detecting, through PCR amplification, a mutation occurring at position 1 or 2 of the 12th codon of the K-ras oncogene. As previously noted, mutations at the first or second position of the 12th codon of K-ras are often associated with certain cancers such as adenocarcinomas of the lung, pancreas, and colon.

Referring to Figures 1A and 1B, the DNA from the patient sample is separated into two strands (A and B), which represent the sense and antisense strands. The DNA represents an oncogene having a point mutation which occurs on the same codon (i.e., codon 12) at position 1 (X₁). The allele-specific primers used to detect the mutation at position 1, include a set of four P1 sense primers (P1-A), each of which is unique with respect to the others. The four P1-A primers are complementary to a gene sequence contiguous with the site of the mutation on

strand A. The four P1-A primers preferably differ from each other only at the terminal 3' nucleotide which is complementary to the wild type nucleotide or to one of the three possible mutations which can occur at this known position. Only the P1-A primer which is fully complementary to the mutation-containing segment on the allele will anneal and extend during amplification.

A common downstream primer (P1-B), complementary to a segment of the B strand downstream with respect to the position of the P1-A primers, is used in combination with each of the P1-A primers. The P1-B primer illustrated in Figure 1 anneals to the allele and is extended during the PCR. Together, the P1-A and P1-B primers identified in Table 1 and illustrated in Figure 1B amplify a fragment of the oncogene having 161 base pairs.

Figures 2A and 2B illustrate a scheme utilizing an additional set of four unique, allele specific primers (P2-A) to detect a mutation which can occur at codon 12 of the oncogene, at position 2 (X_2). The amplification strategy illustrated in Figures 1A and 1B would be used in combination with that illustrated in Figures 2A and 2B to detect mutations at either position 1 (X_1) or position 2 (X_2) in Codon 12.

Referring to Figures 2A and 2B, a set of four unique allele specific primers (P2-A) are used to detect a mutation present at a position 2 (X_2) of codon 12. The four P2-A primers are complementary to

the genetic sequence contiguous with the site of the second possible mutation. These four primers are unique with respect to each other and preferably differ only at the terminal 3' nucleotide which is complementary to the wild type nucleotide or to one of the three possible mutations which can occur at the second known position (X₂).

A single common upstream primer (P2-B) complementary to a segment of the A strand upstream of the mutation, is used in combination with each of the unique P2-A primers. The P2-A and P2-B primers identified in Table 1 and illustrated in Figure 2B will amplify a fragment having 146 base pairs.

During the amplification procedure, the polymerase chain reaction is allowed to proceed for about 20 to 40 cycles and most preferably for 30 cycles. Following an initial denaturation period of about 5 minutes, each cycle, using the AmpliTaq DNA polymerase, typically includes about one minute of denaturation at 95° C, two minutes of primer annealing at about 58° C, and a one minute extension at 72° C. While the temperatures and cycle times noted above are currently preferred, it is noted that various modifications may be made. Indeed, the use of different DNA polymerases and/or different primers may necessitate changes in the amplification conditions. One skilled in the art will readily be able to optimize the amplification conditions.

Exemplary DNA primers which are useful in practicing the method of this invention to detect the K-ras gene, having point mutations at either the first or second position in codon 12 of the gene, are illustrated in Table 1.

TABLE 1

Primers Used to Amplify (by PCR) Position 1
and 2 Mutations at Codon 12 of K-ras Gene
(5'-3')

<u>Sequence*</u>	<u>Strand</u>	<u>P1 or P2</u>
GTGGTAGTTGGAGCTG	A	P1
GTGGTAGTTGGAGCT <u>C</u>	A	P1
GTGGTAGTTGGAGCT <u>T</u>	A	P1
GTGGTAGTTGGAGCT <u>A</u>	A	P1
CAGAGAAACCTTTATCTG	B	P1
ACTCTTGCCTACGCCAC	A	P2
ACTCTTGCCTACGCCAG	A	P2
ACTCTTGCCTACGCCAT	A	P2
ACTCTTGCCTACGCCAA	A	P2
GTACTGGTGGAGTATTT	B	P2

*Underlined bases denote mutations.

The primers illustrated in Table 1 are, of course, merely exemplary. Various modifications can be made to these primers as is understood by those having ordinary skill in the art. For example, the primers could be lengthened or shortened, however the 3' terminal nucleotides must remain the same. In

addition, some mismatches 3 to 6 nucleotides back from the 3' end may be made and would not be likely to interfere with efficacy. The common primers can also be constructed differently so as to be complementary to a different site, yielding either a longer or shorter amplified product.

In one embodiment, the length of each allele specific primer can be different, making it possible to combine multiple allele specific primers with their common distant primer in the same PCR reaction. The length of the amplified product would be indicative of which allele specific primer was being utilized with the amplification. The length of the amplified product would indicate which mutation was present in the specimen.

The primers illustrated in Table 1 and Figures 1B and 2B, and others which could be used, can be readily synthesized by one having ordinary skill in the art. For example, the preparation of similar primers has been described by Stork et al., Oncogene, 6:857-862, 1991.

Other amplification methods and strategies may also be utilized to detect gene sequences in biological fluids according to the method of the invention. For example, another approach would be to combine PCR and the ligase chain reaction (LCR). Since PCR amplifies faster than LCR and requires fewer copies of target DNA to initiate, one could use PCR as first step and then proceed to LCR. Primers such as the common primers used in the allele specific amplification described previously which span a sequence of approximately 285 base pairs in

length, more or less centered on codon 12 of K-ras, could be used to amplify this fragment, using standard PCR conditions. The amplified product (approximately a 285 base pair sequence) could then be used in a LCR or ligase detection reaction (LDR) in an allele specific manner which would indicate if a mutation was present. Another, perhaps less sensitive, approach would be to use LCR or LDR for both amplification and allele specific discrimination. The later reaction is advantageous in that it results in linear amplification. Thus the amount of amplified product is a reflection of the amount of target DNA in the original specimen and therefore permits quantitation.

LCR utilizes pairs of adjacent oligonucleotides which are complementary to the entire length of the target sequence (Barany F., PNAS 88: 189-193, 1991; Barany F., PCR Methods and Applications 1: 5-16, 1991). If the target sequence is perfectly complementary to the primers at the junction of these sequences, a DNA ligase will link the adjacent 3' and 5' terminal nucleotides forming a combined sequence. If a thermostable DNA ligase is used with thermal cycling, the combined sequence will be sequentially amplified. A single base mismatch at the junction of the oligonucleotides will preclude ligation and amplification. Thus, the process is allele specific. Another set of oligonucleotides with 3' nucleotides specific for the mutant would be used in another reaction to identify the mutant allele. A series of standard conditions could be used to detect all possible mutations at any known

site. LCR typically utilizes both strands of genomic DNA as targets for oligonucleotide hybridization with four primers, and the product is increased exponentially by repeated thermal cycling.

A variation of the reaction is the ligase detection reaction (LDR) which utilizes two adjacent oligonucleotides which are complementary to the target DNA and are similarly joined by DNA ligase (Barany F., PNAS 88:189-193, 1991). After multiple thermal cycles the product is amplified in a linear fashion. Thus the amount of the product of LDR reflects the amount of target DNA. Appropriate labeling of the primers allows detection of the amplified product in an allele specific manner, as well as quantitation of the amount of original target DNA. One advantage of this type of reaction is that it allows quantitation through automation (Nickerson et al., PNAS 87: 8923-8927, 1990).

Examples of suitable oligonucleotides for use with LCR for allele specific ligation and amplification to identify mutations at position 1 in codon 12 of the K-ras gene are illustrated below in Table 2.

TABLE 2

Oligonucleotides (5'-3') for use in LCR

<u>Sequence*</u>	<u>Strand</u>	<u>P1 or P2</u>
AGCTCCA ^A CTACCACAAGTT	A1	A
GCACTCTTGCCTACGCCACC	A2-A	A
GCACTCTTGCCTACGCCACA <u>A</u>	A2-B	A
GCACTCTTGCCTACGCCAC <u>G</u>	A2-C	A
GCACTCTTGCCTACGCCACT <u>I</u>	A2-D	A
GGTGGCGTAGGCAAGAGTGC	B1	B
AACTTGTGGTAGTTGGAGCT	B2-A	B
AACTTGTGGTAGTTGGAGCA <u>A</u>	B2-B	B
AACTTGTGGTAGTTGGAGC <u>C</u>	B2-C	B
AACTTGTGGTAGTTGGAGC <u>G</u>	B2-D	B

*Underlined bases denote mutations.

During an amplification procedure involving LCR four oligonucleotides are used at a time. For example, oligonucleotide A1 and, separately, each of the A2 oligonucleotides are paired on the sense strand. Also, oligonucleotide B1 and, separately, each of the B2 oligonucleotides are paired on the antisense strand. For an LCD procedure, two oligonucleotides are paired, i.e., A1 with each of the A2 oligonucleotides, for linear amplification of the normal and mutated target DNA sequence.

The method of the invention is applicable to the detection and quantitation of other oncogenes in DNA present in various biological fluids. The p53 gene is a gene for which convenient detection and quantitation could be useful because alterations in this gene are the most common genetic anomaly in human cancer, occurring in cancers of many histologic types arising from many anatomic sites. Mutations of the p53 may occur at multiple codons within the gene but 80% are localized within 4 conserved regions, or "hot spots", in exons 5, 6, 7 and 8. The most popular current method for identifying the mutations in p53 is a multistep procedure. It involves PCR amplification of exons 5-8 from genomic DNA, individually, in combination (i.e., multiplexing), or sometimes as units of more than one exon. An alternative approach is to isolate total cellular RNA, which is transcribed with reverse transcriptase. A portion of the reaction mixture is subjected directly to PCR to amplify the regions of p53 cDNA using a pair of appropriate oligonucleotides as primers. These two types of amplification are followed by single strand conformation polymorphism analysis (SSCP) which will identify amplified samples with point mutations from normal DNA by differences in mobility when electrophoresed in polyacrylamide gel. If a fragment is shown by SSCP to contain a mutation, the latter is amplified by asymmetric PCR and the sequence determined by the dideoxy-chain termination method (Murakami et al, Can. Res., 51: 3356-33612, 1991).

Further, the ligase chain reaction (LCR) may be useful with p53 since LCR is better able to evaluate multiple mutations at the same time. After determining the mutation, allele specific primers can be prepared for subsequent quantitation of the mutated gene in the patient's plasma at multiple times during the clinical course.

Preferably, the method of the invention is conducted using biological fluid samples of approximately 5ml. However, the method can also be practiced using smaller sample sizes in the event that specimen supply is limited. In such case, it may be advantageous to first amplify the DNA present in the sample using the common primers. Thereafter, amplification can proceed using the allele specific primers.

The method of this invention may be embodied in diagnostic kits. Such kits may include reagents for the isolation of DNA as well as sets of primers used in the detection method, and reagents useful in the amplification. Among the reagents useful for the kit is a DNA polymerase used to effect the amplification. A preferred polymerase is Thermus aquaticus DNA polymerase available from Perkin-Elmer as AmpliTaq DNA polymerase. For quantitation of the mutated gene sequences, the kit can also contain samples of mutated DNA for positive controls as well as tubes for quantitation by competitive PCR having the engineered sequence in known amounts.

The quantitation of the mutated K-ras sequences may be achieved using either slot blot Southern hybridization or competitive PCR. Slot blot Southern hybridization can be performed utilizing the allele specific primers as probes under relatively stringent conditions as described by Verlaan-de Vries et al., Gene 50:313-20, 1986. The total DNA extracted from 5 ml of plasma will be slot blotted with 10 fold serial dilutions, followed by hybridization to an end-labeled allele specific probe selected to be complementary to the known mutation in the particular patient's tumor DNA as determined previously by screening with the battery of allele specific primers and PCR and LCR. Positive autoradiographic signals will be graded semiquantitatively by densitometry after comparison with a standard series of diluted DNA (1-500 ng) from tumor cell cultures which have the identical mutation in codon 12 of the K-ras, prepared as slot blots in the same way.

A modified competitive PCR (Gilliland et al., Proc. Nat. Acad. Sci., USA 87:2725:79; 1990; Gilliland et al., "Competitive PCR for Quantitation of MRNA", PCR Protocols (Acad. Press), pp. 60-69, 1990) could serve as a potentially more sensitive alternative to the slot blot Southern hybridization quantitation method. In this method of quantitation, the same pair of primers are utilized to amplify two DNA templates which compete with each other during the amplification process. One template is the sequence of interest in unknown amount, i.e. mutated K-ras, and the other is an engineered deletion mutant

in known amount which, when amplified, yields a shorter product which can be distinguished from the amplified mutated K-ras sequence. Total DNA extracted from the plasma as described above will be quantitated utilizing slot blot Southern hybridization, utilizing a radiolabelled human repetitive sequence probe (BLUR8). This will allow a quantitation of total extracted plasma DNA so that the same amount can be used in each of the PCR reactions. DNA from each patient (100 ng) will be added to a PCR master mixture containing P1 or P2 allele specific primers corresponding to the particular mutation previously identified for each patient in a total volume of 400 μ l. Forty μ l of master mixture containing 10 ng of plasma DNA will be added to each of 10 tubes containing 10 μ l of competitive template ranging from 0.1 to 10 attomoles. Each reaction mixture will contain dNTPs (25 μ M final concentration including [α - 32 P]dCTP at 50 μ Ci/ml), 50 pmoles of each primer, 2mM MgCl₂, 2 units of T. aquaticus DNA polymerase, 1 x PCR buffer, 50 μ g/ml BSA, and water to a final volume of 40 μ l. Thirty cycles of PCR will be followed by electrophoresis of the amplified products. Bands identified by ethidium bromide will excised, counted and a ratio of K-ras sequence to deletion mutant sequence calculated. To correct for difference in molecular weight, cpm obtained for genomic K-ras bands will multiplied by 141/161 or 126/146, depending upon whether position 1 (P1) or position 2 (P2) primers are used. (The exact ratio will depend upon the length of the deletion mutant.) Data will be plotted as log ratio of deletion template DNA/K-ras DNA vs. log input deletion template DNA (Gilliland et al. 1990a, 1990b).

A modified competitive PCR could also be developed in which one primer has a modified 5' end which carries a biotin moiety and the other primer has a 5' end with a fluorescent chromophore. The amplified product can then be separated from the reaction mixture by adsorption to avidin or streptavidin attached to a solid support. The amount of product formed in the PCR can be quantitated by measuring the amount of fluorescent primer incorporated into double-stranded DNA by denaturing the immobilized DNA by alkali and thus eluting the fluorescent single stands from the solid support and measuring the fluorescence (Landgraf et al., Anal. Biochem. 182:231-235, 1991).

The competitive template preferably comprises engineered deletion mutants with a sequence comparable to the fragments of the wild type K-ras and the mutated K-ras gene amplified by the P1 and P2 series of primers described previously, except there will be an internal deletion of approximately 20 nucleotides. Therefore, the amplified products will be smaller, i.e., about 140 base pairs and 125 base pairs when the P1 primers and P2 primers are used, respectively. Thus, the same primers can be used and yet amplified products from the engineered mutants can be readily distinguished from the amplified genomic sequences.

Eight deletion mutants will be produced using the polymerase chain reaction (Higuchi et al., Nucleic Acids Res. 16:7351-67 1988); Vallette et al., Nucleic Acids Res. 17:723-33, 1989; Higuchi,

PCR Technology, Ch. 6, pp. 61-70 (Stockton Press, 1989)). The starting material will be normal genomic DNA representing the wild-type K-ras or tumor DNA from tumors which are known to have each of the possible point mutations in position one and two of codon 12. The wild-type codon 12 is GGT. The following tumor DNA can be used:

First position codon 12 mutations

G-A A549
G-T* Calu1, PR371
G-C A2182, A1698

Second position codon 12 mutations

G-A* Aspcl
G-T* SW480
G-C 818-1, 181-4, 818-7

(*G-T transversions in the first or second position account for approximately 80% of the point mutations found in pulmonary carcinoma and GAT (aspartic acid) or GTT (valine) are most common in pancreatic cancer.

The deletion mutants with an approximately 20 residue deletion will be derived as previously described (Vallette et al. 1989). In summary, the P1 and P2 primers will be used in an allele specific manner with the normal DNA or with DNA from the tumor cell line with each specific mutation. Each of these would be paired for amplification with a common primer which contains the sequence of the common

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primer normally used with either the P1 and P2 allele specific primers, i.e., "P1-B" or "P2-B" at the 5' end with an attached series of residues representing sequences starting approximately 20 bases downstream, thus spanning the deleted area (common deletion primer 1 and 2, CD1 and CD2). The precise location and therefore sequence of the 3' portion of the primer will be determined after analysis of the sequence of the ras gene in this region with OLIGO (NBI, Plymouth, MN), a computer program which facilitates the selection of optimal primers. The exact length of the resultant amplified product is not critical, so the best possible primer which will produce a deletion of 20-25 residues will be selected. For example, with P2 primers the allele specific primer for the wild-type will be 5' ACTCTTGCCCTACGCCAC 3' complementary to residues 35 to 51 in the coding sequence. To effect a deletion of approximately 20 residues in the complementary strand, the common upstream primer to be used with the wild-type and the three allele specific primers for mutations in position two of codon 12 will be 40 residues long (CD2) complementary to residues -95 to -78 (the currently preferred common upstream primer for use with P2 allele specific primers and residues at approximately -58 to -25). The amplified shorter product will be size-separated by gel electrophoresis and purified by Prep-a-Gene (Biorad). DNA concentrations will be determined by the ethidium bromide staining with comparison to dilutions of DNA of known concentration. This approach will be repeated eight times, using the four P1 primers and common primer (CD1) constructed as above, and four

times with the four P2 primers and common primer (CD2). These deletion mutants will be amplified, using the same allele specific primers used to amplify the genomic DNA. Therefore, they can be used subsequently in known serial dilutions in a competitive PCR, as outlined above.

The invention is further illustrated by the following non-limiting examples.

Example 1

Blood was collected in 13 x 75 mm vacutainer tubes containing 0.05 ml of 15% K₃EDTA. The tubes were immediately centrifuged at 4°C for 30 minutes at 1000 g, the plasma was removed and recentrifuged at 4°C for another 30 minutes at 1000 g.

The plasma was stored at -70°C. Next, DNA was deproteinized by adding an equal volume of 20% NaCl to 5 ml aliquots of plasma which were then boiled for 3 to 4 minutes. After cooling, the samples were centrifuged at 3000 rpm for 30 minutes. The supernatant was removed and dialysed against three changes of 10 mM Tris-HCl (pH 7.5)/1 mM EDTA (pH 8.0) ("TE") for 18 to 24 hours at 4°C. The DNA was extracted once with two volumes of phenol, 2x1 volume phenol:chloroform: isoamyl alcohol (25:24:1) and 2x1 volume chloroform: isoamyl alcohol (24:1). DNA was subsequently precipitated with NaCl at 0.3M, 20µg/ml glycogen as a carrier and 2.5 volumes of 100% ethanol at minus 20°C for 24 hours. DNA was recovered by centrifugation in an Eppendorf Centrifuge at 4°C for 30 minutes. The DNA was then resuspended in a TE buffer. The DNA extracted and prepared in the above manner was then able to be amplified.

Example 2

An allele specific amplification of DNA obtained and prepared according to example 1 was conducted by PCR as follows to detect the K-ras gene in the DNA having a mutation at position 1 or 2 of the codon 12 of the K-ras gene. In each of eight reaction tubes was added DNA extracted from 0.5 ml of plasma in total volume of 40 μ l containing 67 mM Tris-HCl (pH 8.8), 10 mM β -mercaptoethanol, 16.6 μ M ammonium sulfate, 6.7 μ M EDTA, 2.0mM, $MgCl_2$, 50 μ g/ml BSA, 25 μ M dNTP. Also, 50 pmoles of each of the primers identified in Table 1 was included, together with 3 units of Thermus aquaticus DNA polymerase (available from Perkin-Elmer as AmpliTaq). PCR was conducted with an initial denaturation at 95°C for 5 minutes, followed by 30 cycles of PCR amplification in a DNA thermal cycler (Cetus; Perkin-Elmer Corp. Norwalk, Connecticut). Each amplification cycle includes a 1 minute denaturation at 95°C, a 2 minute primer annealing period at 58°C, and a 1 minute extension period at 72°C.

Following the completion of amplification, 10-15 μ l of each of the PCR reaction products is analyzed by electrophoresis in a 2% agarose gel/1X TAE-0.5 μ g/ml EtBr. The electrophoresis uses an applied voltage of 100 volts for 90 minutes. Photographs of the samples are then taken using ultraviolet light under standard conditions.

It is understood that various modifications can be made to the present invention without departing from the scope of the claimed invention.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Sorenson, George D.
- (ii) TITLE OF INVENTION: Detection of
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In Biological
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- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy Disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: ASCII Text
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 - (A) APPLICATION NUMBER:
 - (B) FILING DATE: 27 APR - 1992
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- (viii) ATTORNEY/AGENT INFORMATION:
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 - (C) REFERENCE/DOCKET NUMBER: DCI-037
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- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1

- (2) INFORMATION FOR SEQ ID NO:1:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA
 - (iii) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GTGGTAGTTG GAGCTG 16

- (2) INFORMATION FOR SEQ ID NO:2:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA
 - (iii) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GTGGTAGTTG GAGCTC 16

- (2) INFORMATION FOR SEQ ID NO:3:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 16 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA
 - (iii) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 16 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GTGGTAGTTG GAGCTA

16

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CAGAGAAACC TTTATCTG

18

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 17 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ACTCTTGCCT ACGCCAC

17

- (2) INFORMATION FOR SEQ ID NO:7:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA
 - (iii) SEQUENCE DESCRIPTION: SEQ ID NO:7:

ACTCTTGCCT ACGCCAG

17

- (2) INFORMATION FOR SEQ ID NO:8:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA
 - (iii) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ACTCTTGCCT ACGCCAA

17

- (2) INFORMATION FOR SEQ ID NO:9:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA
 - (iii) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ACTCTTGCCT ACGCCAT

17

- (2) INFORMATION FOR SEQ ID NO:10:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA
 - (iii) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GTACTGGTGG AGTATTT 17

- (2) INFORMATION FOR SEQ ID NO:11:
- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA
 - (iii) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGCTCCAACT ACCACAAGTT 20

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- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
 - (ii) MOLECULE TYPE: DNA
 - (iii) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GCACTCTTGC CTACGCCACC 20

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:13:

GCACTCTTGC CTACGCCACA

20

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(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GCACTCTTGC CTACGCCACG

20

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:15:

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20

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:16:

GGTGGCGTAG GCAAGAGTGC

20

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:17:

AACTTGTGGT AGTTGGAGCT

20

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 20 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:18:

AACTTGTGGT AGTTGGAGCA

20

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(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AACTTGTGGT AGTTGGAGCC

20

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AACTTGTGGT AGTTGGAGCG

20

Claims:

1. A method of detecting a mutant allele, comprising the steps of:

providing a sample of a biological fluid containing soluble DNA, including a mutant allele of interest;

extracting the DNA from the sample;

denaturing the DNA to free first and second strands of the DNA;

amplifying the mutant allele of interest in an allele specific manner using at least a first set of four allele specific oligonucleotide primers having one primer complementary to a mutation-containing segment on a first strand of the DNA and a first common primer for pairing during amplification to each allele specific primer, the common primer being complementary to a segment of a second strand of the DNA distant with respect to the position of the first primer; and

detecting the presence of the mutant allele of interest.

2. The method of claim 1 further comprising the step of removing protein from the sample and inactivating any DNase within the sample before the step of extracting the DNA.

3. The method of claim 2, wherein the mutant allele is amplified in an allele specific manner using the polymerase chain reaction (PCR).

4. The method of claim 3, wherein following the amplification step, the step of detecting the presence of the mutant allele of interest comprises performing an allele specific ligase chain reaction (LCR) or a ligase detection reaction (LDR) using the amplified product of PCR.

5. The method of claim 2 wherein protein is removed and DNases are inactivated by adding a salt solution to the sample and subsequently boiling the sample.

6. The method of claim 2 wherein the biological fluid is selected from the group consisting of whole blood, serum, plasma, urine, sputum, and cerebral spinal fluid.

7. The method of claim 2 wherein the mutant allele comprises a gene sequence having a point mutation at a known location.

8. The method of claim 7 wherein the first DNA strand is the sense strand and the second DNA strand is the antisense strand.

9. The method of claim 2 wherein the step of amplifying the mutant allele with the PCR is conducted using a DNA polymerase which lacks the 3' exonuclease activity and therefore the ability to repair single nucleotide mismatches at the 3' end of the primer.

10. The method of claim 9 wherein the DNA polymerase is a Thermus aquaticus DNA polymerase.

11. The method of claim 9 wherein the first set of allele specific oligonucleotide primers comprises:

four sense primers, one of which has a 3' terminal nucleotide complementary to a point mutation of the sense strand, and the remaining three of which are complementary to the wild type sequence for the segment to be amplified and to sequences having the remaining two possible mutations at the mutated point of the sense strand; and

a common antisense primer complementary to a segment of the antisense strand distant from the location on the sense strand at which the sense primers will anneal, the common antisense primer being paired with each of the sense primers during amplification.

12. The method of claim 11 wherein the 3' terminal nucleotide of the complementary sense primer anneals with the mutated nucleotide of the sense strand.

13. The method of claim 3 wherein the mutant allele comprises a gene sequence having a point mutation at one of two known locations.

14. The method of claim 13 wherein the step of amplifying the mutant allele through the PCR further comprises the use of a second set of four allele specific oligonucleotide primers, in conjunction with the first set, wherein the second set of allele specific oligonucleotide primers comprises:

four sense primers, one of which has a 3' terminal nucleotide complementary to a point mutation of the sense strand, and the remaining three of which are complementary to the wild type sequence for the segment to be amplified and sequences having the remaining two possible mutations at the mutated point of the sense strand; and

a common antisense primer complementary to a segment of the antisense strand distant from the location on the sense strand at which the sense primers will anneal, the common antisense primer being paired with each of the sense primers during amplification.

15. The method of claim 14 wherein the 3' terminal nucleotide of the complementary sense primer anneals with the mutated nucleotide of the sense strand.

16. The method of claim 15 wherein the mutant allele to be detected is the K-ras gene sequence having a mutation at position 1 or 2 in the twelfth codon.

17. The method of claim 16 wherein the first set of allele specific oligonucleotide primers comprises sense primers having the following sequences

5'GTGGTAGTTGGAGCTG 3' (wild type)
5'GTGGTAGTTGGAGCTC 3'
5'GTGGTAGTTGGAGCTT 3'
5'GTGGTAGTTGGAGCTA 3'

and the common antisense primer having the following sequence

5'CAGAGAAACCTTTATCTG 3'.

18. The method of claim 14 wherein the second set of allele specific oligonucleotide primers comprises sense primers having the following sequences

5'ACTCTTGCCTACGCCAC 3' (wild type)
5'ACTCTTGCCTACGCCAG 3'
5'ACTCTTGCCTACGCCAT 3'
5'ACTCTTGCCTACGCCAA 3'

and the common antisense primer having the following sequence

5'GTACTGGTGGAGTATTT 3'.

19. The method of claim 2 wherein the step of detecting the presence of amplified DNA is conducted by gel electrophoresis in 1-5% agarose gel.

20. The method of claim 2 wherein the biological fluid is selected from the group consisting of whole blood, serum, plasma, urine, sputum, and cerebral spinal fluid.

21. A diagnostic kit for detecting the presence of a mutated K-ras gene sequence in biological fluid, wherein the mutation is present in the twelfth codon at position 1, comprising:
reagents to facilitate the deproteinization and isolation of DNA;
reagents to facilitate amplification by PCR;
a heat stable DNA polymerase; and
a first set of allele specific oligonucleotide sense primers having the following sequences

5'GTGGTAGTTGGAGCTG 3'

5'GTGGTAGTTGGAGCTC 3'

5'GTGGTAGTTGGAGCTT 3'

5'GTGGTAGTTGGAGCTA 3'

and a first common antisense primer having the following sequence

5'CAGAGAAACCTTTATCTG '3'

22. The diagnostic kit of claim 21 further comprising

a second set of allele specific oligonucleotide sense primers having the following sequences

5'ACTCTTGCCTACGCCAC 3'

5'ACTCTTGCCTACGCCAG 3'

5'ACTCTTGCCTACGCCAT 3'

5'ACTCTTGCCTACGCCAA 3'

and a second common antisense primer having the following sequence

5'GTACTGGTGGAGTATTT 3'

wherein the second set of allele specific oligonucleotide primers and the second common primer are useful in detecting in biological fluid the presence of a mutated K-ras gene sequence in the twelfth codon at position 2.

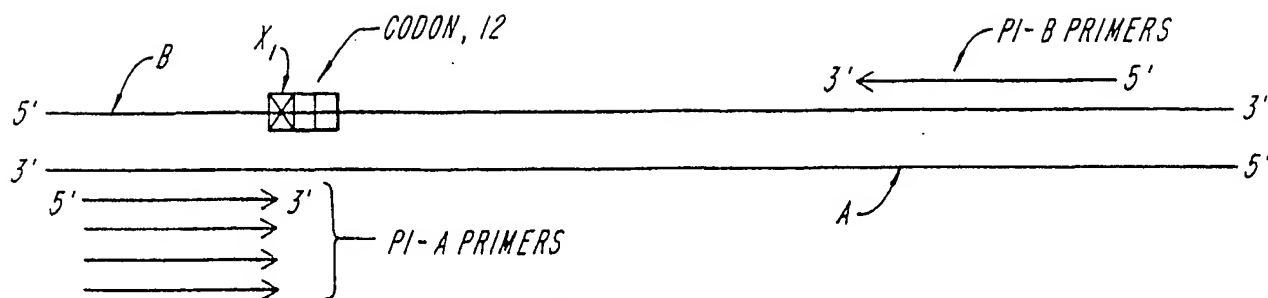


FIG. 1A

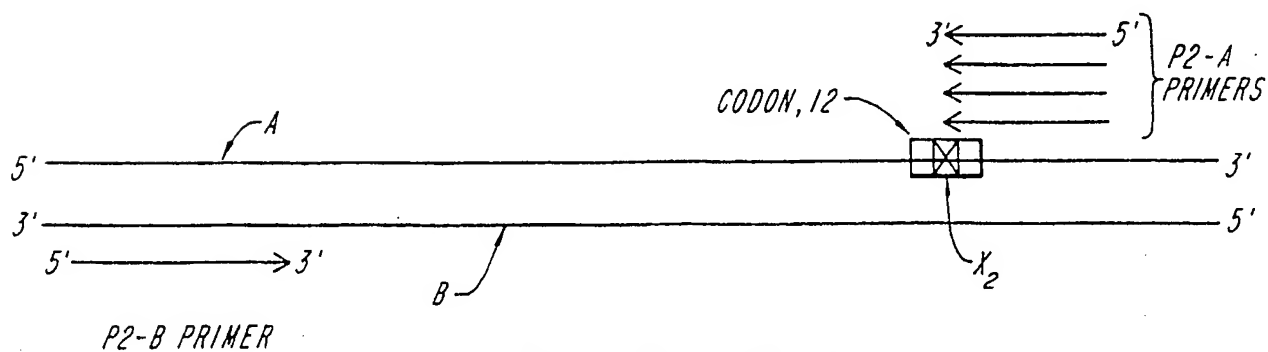
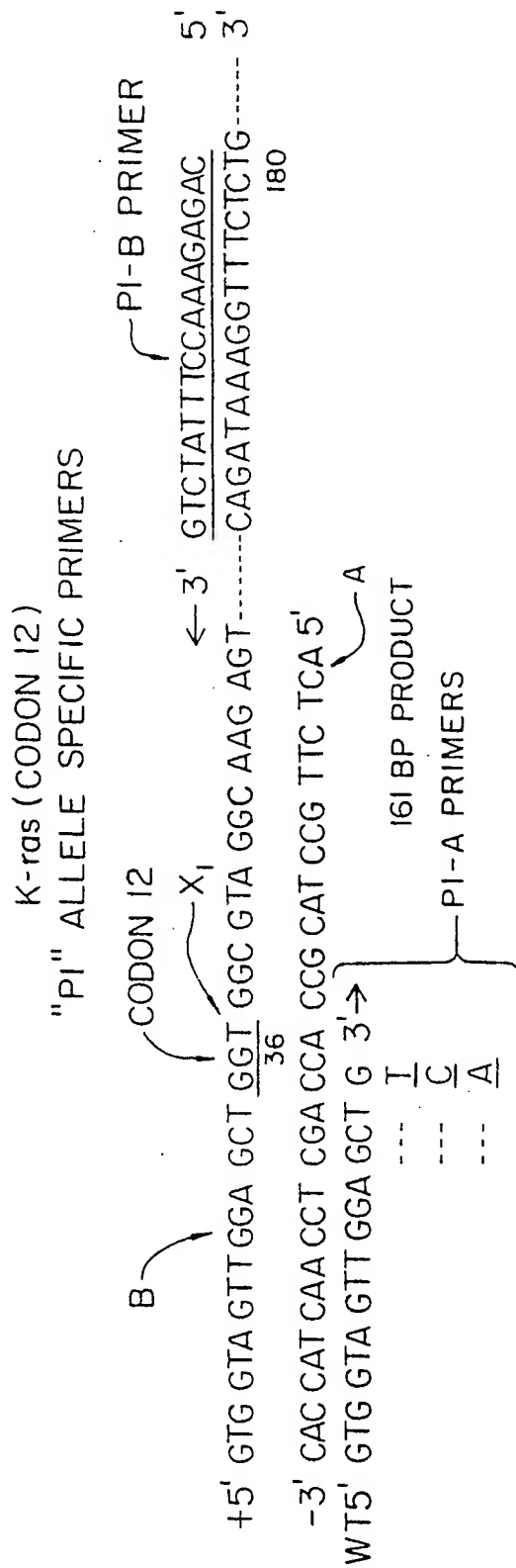


FIG. 2A

**FIG. 1B**

K-ras (CODON 12)
"P2" ALLELE SPECIFIC PRIMERS

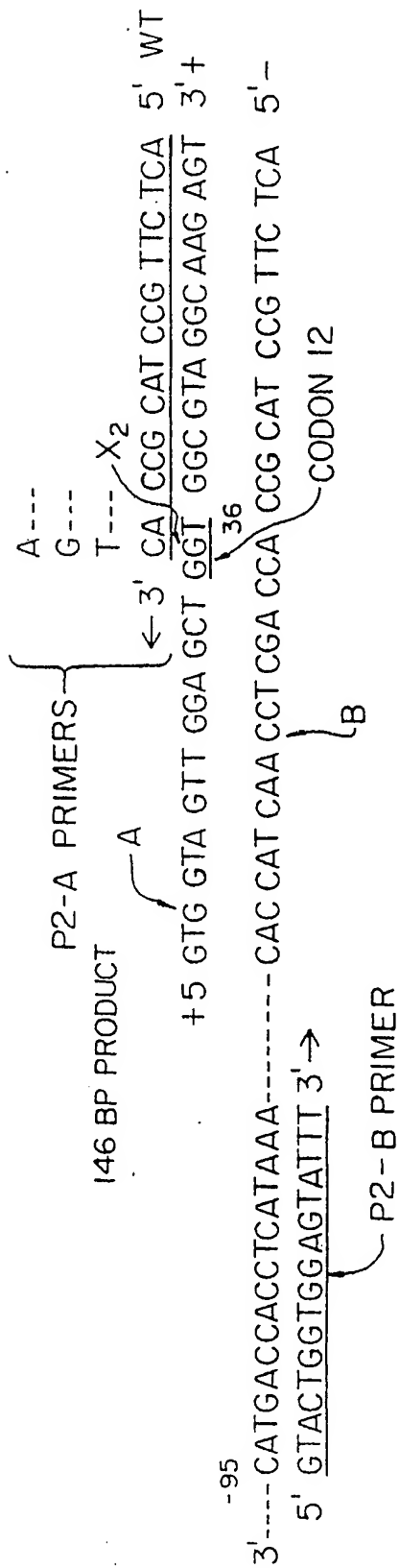


FIG. 2B

A. CLASSIFICATION OF SUBJECT MATTER

IPC(S) : C12Q 1/68; C12P 19/34; C07H 21/04

US CL : 435/6, 91; 536/24.33

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91; 536/24.33; 935/78

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, EMBASE, MEDLINE, SCISEARCH, EMBL, GENBANK

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ONCOGENE, Volume 6, issued May 1991 by MacMillan Press Ltd., P. Stork et al., "Detection of K-ras mutations in pancreatic and hepatic neoplasms by non-isotopic mismatched polymerase chain reaction", pages 857-862, see entire document.	1-22
Y	WO, 89/00206 (BALAZS ET AL) 12 NOVEMBER 1989, see entire document.	1-20
Y	US, A, 4,988,617 (LANDEGREN ET AL) 29 JANUARY 1991, see entire document.	1-22
A,P	US, A, 5,137,806 (LeMAISTRE ET AL) 11 AUGUST 1992, see entire document.	1-22



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	*T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be part of particular relevance	*X*	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
E earlier document published on or after the international filing date	*Y*	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*Z*	document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means		
P document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search

08 JUNE 1993

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JUL 13 1993

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(54) Rapid detection of nucleic acid sequences in a sample by labeling the sample.

(57) A method for detecting one or more microorganisms or polynucleotide sequences from eukaryotic sources in a nucleic acid-containing test sample comprising

- (a) preparing a test sample comprising labeling the nucleic acids in the test sample,
- (b) preparing one or more process by immobilizing a single-stranded nucleic acid of one or more known microorganisms or sequences from eukaryotic sources,
- (c) contacting, under hybridization conditions, the labeled single-stranded nucleic acid to form hybridized labeled nucleic acids, and
- (d) assaying for the hybridized nucleic acids by detecting the label. The method can be used to detect genetic disorders, e.g., sickle-cell anemia.

EP 0 235 726 A2

RAPID DETECTION OF NUCLEIC ACID SEQUENCES IN A SAMPLE BY LABELING THE SAMPLE

BACKGROUND OF THE INVENTION

Field of the Invention

The present application relates to the detection and identification of microorganisms and the detection and identification of particular prokaryotic or eukaryotic DNA sources in a nucleic acid containing test sample.

Still further, the present invention relates to a method for the lysis of whole cells.

Background Information

A. The Detection of Microorganisms

The identification of species of microorganisms in a sample containing a mixture of microorganisms, by immobilizing the DNA from the sample and subjecting it to hybridization with a labelled specimen of species -specific DNA from a known microorganism and observing whether hybridization occurs between the immobilized DNA and the labelled specimen, has been disclosed in PCT patent application No. PCT/US83/01029.

The most efficient and sensitive method of detection of nucleic acids such as DNA after hybridization requires radioactively labeled DNA. The use of autoradiography and enzymes prolongs the assay time and requires experienced technicians.

U.S.P. 4,358,535 to Falkow et al describe infectious disease diagnosis using labeled nucleotide probes complementary to nucleic acid coding for a characteristic pathogen product.

B. The Detection of Specific Eukaryotic Sequences

The identification of specific sequence alteration in an eukaryotic nucleic acid sample by immobilizing the DNA from the sample and subjecting it to hybridization with a labeled oligonucleotide and observing whether hybridization occurs between the immobilized DNA and the labeled probe, has been described in EP -patent application No. 86 117 978 filed December 23, 1986, now pending.

It is known that the expression of a specific gene determines the physical condition of a human being. For example, a change in the beta-globin gene coding sequence from GAG to GTG at the sixth amino acid position produces sickle-beta-globin and a homozygote can have a disease known as sickle cell anemia. Similarly deletion of particular sequences from alpha-globin or beta-globin genes can cause thalassemias. A recent survey, The New Genetics and Clinical Practice, D.J. Weatherall, The Nuffield Provincial Hospitals Trust, (1982), chapter 2 describes the frequency and clinical spectrum of genetic diseases.

Problems associated with genetic defects can be diagnosed by nucleic acid sequence information. The easiest way to detect such sequence information is to use the method of hybridization with a specific probe of a known sequence.

U.S.P. 4,395,486 to Wilson et al describe a method for the direct analysis of sickle cell anemia using a restriction endonuclease assay.

Edward M. Rubin and Yuet Wai Kan, "A Simple Sensitive Prenatal Test for Hydrops Fetalis Caused By α -Thalassaemia", The Lancet, January 12, 1985, pp. 75-77 describes a dot blot analysis to differentiate between the genotypes of homozygous alpha-thalassemia and those of the haemoglobin-H disease and alpha-thalassemia trait.

The most efficient and sensitive method of detection of nucleic acids, such as DNA, after hybridization requires radioactively labelled DNA. The use of autoradiography and enzymes prolongs the assay time and requires experienced technicians.

Recently, a non-radioactive method of labelling DNA was described by Ward et al, European Patent Application 63,879. Ward et al, use the method of nick translation to introduce biotinylated U (uracil) residues into DNA, replacing T (thymine). The biotin residue is then assayed with antibiotin antibody or an avidin-containing system. The detection in this case is quicker than autoradiography, but the nick translation

method requires highly skilled personnel. Moreover, biotinylation using biotinylated UTP (uridine triphosphate) works only for thymine-containing polynucleotides. The use of other nucleoside triphosphates is very difficult because the chemical derivatization of A (adenine) or G (guanine) or C (cytosine) (containing -NH₂) with biotin requires the skills of trained organic chemists.

5

C. Cell Lysis

The present invention also provides a method for the efficient lysis of whole cells such that their DNA is released and made available for photochemical labeling. While eukaryotic cells derived from multicellular animals are easily lysed under relatively mild conditions, single cell eukaryotes and prokaryotes, especially Gram positive prokaryotes, are more difficult to lyse due to the complicated chemical nature and extent of cross-linking of their cell walls. Methods do exist for efficiently lysing these refractory organisms, either by chemical-enzymatic or physical means, but these methods are often complicated, time-consuming and inappropriate for preserving the integrity of DNA.

15

SUMMARY OF THE INVENTION

It is accordingly an object of the present invention to provide a method for detection of microorganisms in a nucleic acid-containing test sample.

20

It is another object of the invention to provide a method for a simultaneous assay for the presence of more than one nucleic acid sequence.

Another object is to provide a method to identify particular prokaryotic or eukaryotic DNA sequences and a method for distinguishing alleles of individual genes.

25

Another object of the invention is to provide a simple photochemical method of labeling the unknown test sample.

A further object of the invention is to label the probes with different kinds of labels so that when the probes are hybridized with an immobilized, unknown, unlabelled test sample, the type of label remaining bound after hybridization and washing, will determine the type of nucleic acid sequence present in the unknown sample.

30

A still further object of the invention is to use whole chromosomal nucleic acid as the probe and/or as the test sample.

Also the invention relates to the use of oligonucleotides as immobilized probes.

These and other objects and advantages are realized in accordance with the present invention for a method of detecting nucleic acid sequences in a nucleic acid-containing test sample.

35

The method involves the following:

(a) preparing a test sample comprising labeling the nucleic acids of the organisms or cells or cell debris in the test sample,

(b) preparing one or more probes by immobilizing a single-stranded DNA or an oligonucleotide of one or more known microorganisms or eukaryotes, or sequences representing particular genes or their alleles,

40

(c) contacting, under hybridization conditions, the labeled single-stranded sample nucleic acid and the immobilized single-stranded (probe) nucleic acid or the immobilized oligonucleotide to form hybridized labeled nucleic acids and

45

(d) assaying for the hybridized nucleic acids by detecting the label.

In the above method, steps (a) and (b) can be reversed.

The method further comprises denaturing the labeled nucleic acids from step (a) to form labeled denatured nucleic acids.

According to the invention, a labeled nucleic acid test sample is contacted simultaneously with several different types of DNA probes for hybridization. The nucleic acid test sample is labeled and hybridized with several unlabeled immobilized probes. The positions of the probes are fixed, and the labeled probe detected after hybridization will indicate that the test sample carries a nucleic acid sequence complementary to the corresponding probe.

50

Nucleic acid probes for several microbiological systems or for different alleles of one or more genes can be immobilized separately on a solid support, for example, nitrocellulose paper. The test sample nucleic acids are labeled and remain in solution. The solid material containing the immobilized probe is brought in contact with the labeled test nucleic acid solution under hybridization conditions. The solid

55

material is washed free of unhybridized nucleic acid and the label is assayed. The presence of the label with one or more of the probes indicates that the test sample contains nucleic acids substantially complementary to those probes and hence originate, for example, from an infection by particular microbiological systems.

5 Labeling can be accomplished in a whole living cell or a cell lysate, and can be non-isotopic. The nucleic acid can be used for hybridization without further purification.

The present invention also concerns specific lysis conditions to release nucleic acids from both gram positive and gram negative bacteria.

10 The present invention further concerns a kit for detecting microorganisms or eukaryotes in a test sample comprising

(a) a support solid containing single-stranded DNA of one or more known microorganisms or eukaryotes immobilized thereon, e.g., a strip containing dots or spots of known microorganisms or eukaryotes,

(b) a reagent for labeling the nucleic acid of the test sample,

15 (c) a reagent for releasing and denaturing DNA in the test sample, and

(d) hybridization reagents.

For chemiluminescence detection of the hybridized nucleic acid, the kit would further comprise a reagent for chemiluminescent detection.

In the above described kit, the reagent for labeling is given hereinbelow in a discussion on labels.

20 Reagents for releasing and denaturing DNA include sodium hydroxide and lysing agents such as detergents and lysozymes.

Typical hybridization reagents includes a mixture of sodium chloride, sodium citrate, SDS (sodium dodecyl sulfate), bovine serum albumin, nonfat milk or dextran sulfate and optionally formamide.

25

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1 is an autoradiograph of results of immobilization of an oligonucleotide sequence specific for hemoglobin mutation.

30 Fig. 2 is a photograph of results of hybridization with labeled genomic DNA for non radioactive detection.

DETAILED DESCRIPTION OF THE INVENTION

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The nucleic acid is preferably labeled by means of photochemistry, employing a photoreactive DNA-binding furocoumarin or a phenanthridine compound to link the nucleic acid to a label which can be "read" or assayed in conventional manner, including fluorescence detection. The end product is thus a labeled nucleic acid comprising (a) a nucleic acid component, (b) an intercalator or other DNA-binding ligand photochemically linked to the nucleic acid component, and (c) a label chemically linked to (b).

40

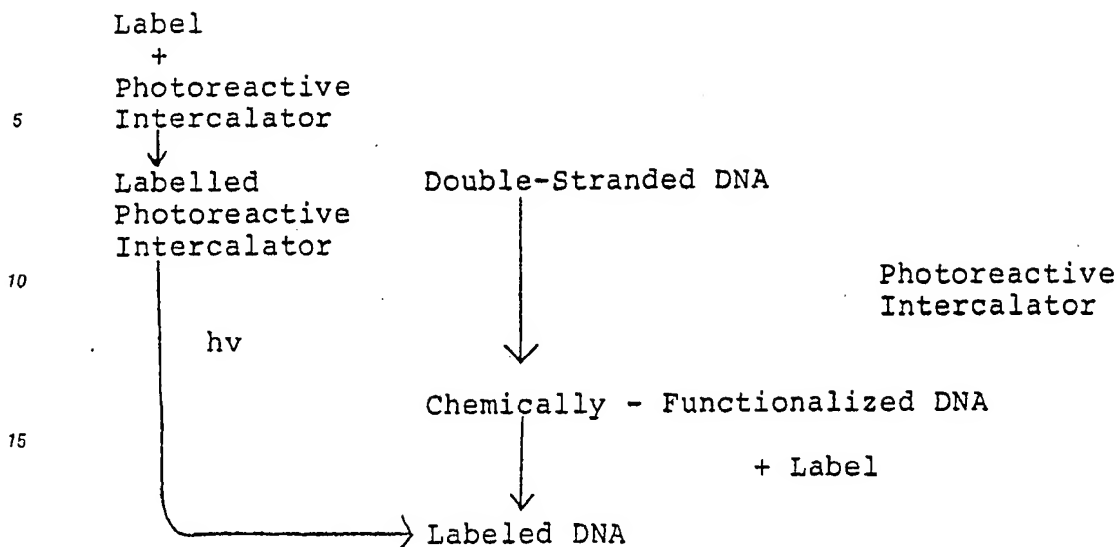
The photochemical method provides more favorable reaction conditions than the usual chemical coupling method for biochemically sensitive substances. The intercalator and label can first be coupled and then photoreacted with the nucleic acid, or the nucleic acid can first be photoreacted with the intercalator and then coupled to the label.

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A general scheme for coupling a nucleic acid, exemplified by double-stranded DNA, to apply a label, is as follows:

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Where the hybridizable portion of the nucleic acid is in a double stranded form, such portion is then denatured to yield a hybridizable single stranded portion. Alternatively, where the labeled DNA comprises the hybridizable portion already in single stranded form, such denaturation can be avoided if desired. Alternatively, double stranded DNA can be labeled by the approach of the present invention after hybridization has occurred using a hybridization format which generates double stranded DNA only in the presence of the sequence to be detected.

To produce specific and efficient photochemical products, it is desirable that the nucleic acid component and the photoreactive intercalator compound be allowed to react in the dark in a specific manner.

For coupling to DNA, aminomethyl psoralen, aminomethyl angelicin and amino alkyl ethidium or methidium azides are particularly useful compounds. They bind to double-stranded DNA and only the complex produces photoadduct. In the case where labeled double-stranded DNA must be denatured in order to yield a hybridizable single stranded region, conditions are employed so that simultaneous interaction of two strands of DNA with a single photoadduct is prevented. It is necessary that the frequency of modification along a hybridizable single stranded portion of the nucleic acid not be so great as to substantially prevent hybridization, and accordingly there preferably will be not more than one site of modification per 25, more usually 50, and preferably 100, nucleotide bases. Angelicin derivatives are superior to psoralen compounds for monoadduct formation. If a single-stranded DNA nucleic acid is covalently attached to some extra double-stranded DNA, use of phenanthridium and psoralen compounds is desirable since these compounds interact specifically to double-stranded DNA in the dark. The chemistry for the synthesis of the coupled reagents to modify nucleic acids for labeling, described more fully hereinbelow, is similar for all cases.

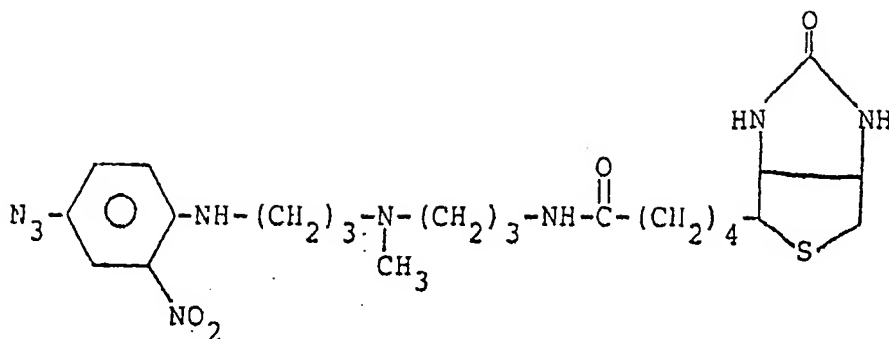
The nucleic acid component can be single or double stranded DNA or RNA or fragments thereof such as are produced by restriction enzymes or even relatively short oligomers.

The DNA-binding ligands of the present invention used to link the nucleic acid component to the label can be any suitable photoreactive form of known DNA-binding ligands. Particularly preferred DNA-binding ligands are intercalator compounds such as the furocoumarins, e.g., angelicin (isopsoralen) or psoralen or derivatives thereof which photochemically will react with nucleic acids, e.g., 4'-aminomethyl-4,5'-dimethyl angelicin, 4'-aminomethyl-trioxsalen (4'-aminomethyl-4,5',8-trimethyl-psoralen), 3-carboxy-5-or -8-amino-or-hydroxy-psoralen, as well as mono-or bis-azido aminoalkyl methidium or ethidium compounds.

Particularly useful photoreactive forms of intercalating agents are the azido-intercalators. Their reactive nitrenes are readily generated at long wavelength ultraviolet or visible light and the nitrenes of arylazides prefer insertion reactions over their rearrangement products (see White et al, Methods in Enzymol., 46, 644 (1977)). Representative intercalating agents include azidoacridine, ethidium monoazide, ethidium dimer azide (Mitchell et al, JACS, 104, 4265 (1982)), 4-azido-7-chloroquinoline, and 2-azidofluorene. A specific nucleic acid binding azido compound has been described by Forster et al, Nucleic Acid Res., 13, (1985), 745. The structure of such compound is as follows:

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Other useful photoreactable intercalators are the furocoumarins which form (2+2) cycloadducts with pyrimidine residues. Alkylating agents can also be used such as bis-chloroethylamines and epoxides or aziridines, e.g., aflatoxins, polycyclic hydrocarbon epoxides, mitomycin and norphillin A.

Nonlimiting examples of intercalator compounds for use in the present invention include acridine dyes, phenanthridines, phenazines, furocoumarins, phenothiazines and quinolines.

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The label which is linked to the nucleic acid component according to the present invention can be any chemical group or residue having a detectable physical or chemical property, i.e., labeling can be conducted by chemical reaction or physical adsorption. The label will bear a functional chemical group to enable it to be chemically linked to the intercalator compound. Such labeling materials have been well developed in the field of immunoassays and in general most any label useful in such methods can be applied to the present invention. Particularly useful are enzymatically active groups, such as enzymes (see *Clin. Chem.*, (1976), 22, 1243), enzyme substrates (see British Pat. Spec. 1,548,741), coenzymes (see U.S. Patent Nos. 4,230,797 and 4,238,565) and enzyme inhibitors (see U.S. Patent No. 4,134,792; fluorescers - (see *Clin. Chem.*, (1979), 25, 353), and chromophores including phycobiliproteins; luminescers such as chemiluminescers and bioluminescers (see *Clin. Chem.*, (1979), 25, 512, and *ibid*, 1531); specifically bindable ligands, i.e., protein binding ligands; and residues comprising radioisotopes such as ^3H , ^{35}S , ^{32}P , ^{125}I , and ^{14}C . Such labels are detected on the basis of their own physical properties (e.g., fluorescers, chromophores and radioisotopes) or their reactive or binding properties (e.g., enzymes, substrates, coenzymes and inhibitors). For example, a cofactor-labeled nucleic acid can be detected by adding the enzyme for which the label is a cofactor and a substrate for the enzyme. A hapten or ligand (e.g., biotin) labeled nucleic acid can be detected by adding an antibody or an antibody pigment to the hapten or a protein (e.g., avidin) which binds the ligand, tagged with a detectable molecule. An antigen can also be used as a label. Such detectable molecule can be some molecule with a measurable physical property - (e.g., fluorescence or absorbance) or a participant in an enzyme reaction (e.g., see above list). For example, one can use an enzyme which acts upon a substrate to generate a product with measurable physical property. Examples of the latter include, but are not limited to, beta-galactosidase, alkaline phosphatase, papain and peroxidase. For *in situ* hybridization studies, ideally the final product is water insoluble. Other labels, e.g., dyes, will be evident to one having ordinary skill in the art.

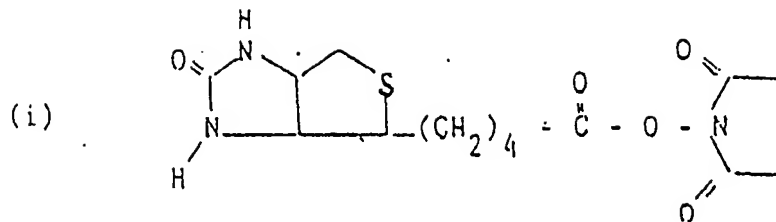
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The label will be linked to the intercalator compound, e.g., acridine dyes, phenanthridines, phenazines, furocoumarins, phenothiazines and quinolines, by direct chemical linkage such as involving covalent bonds, or by indirect linkage such as by the incorporation of the label in a microcapsule or liposome which in turn is linked to the intercalator compound. Methods by which the label is linked to the intercalator compounds are essentially known in the art and any convenient method can be used to perform the present invention.

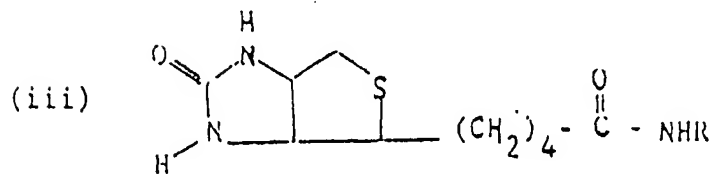
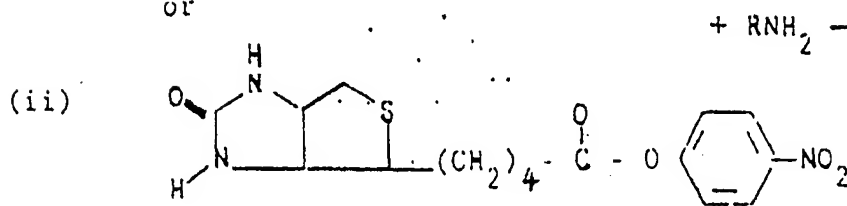
Advantageously, the intercalator compound is first combined with label chemically and thereafter combined with the nucleic acid component. For example, since biotin carries a carboxyl group, it can be combined with a furocoumarin by way of amide or ester formation without interfering with the photochemical reactivity of the furocoumarin or the biological activity of the biotin, e.g.,

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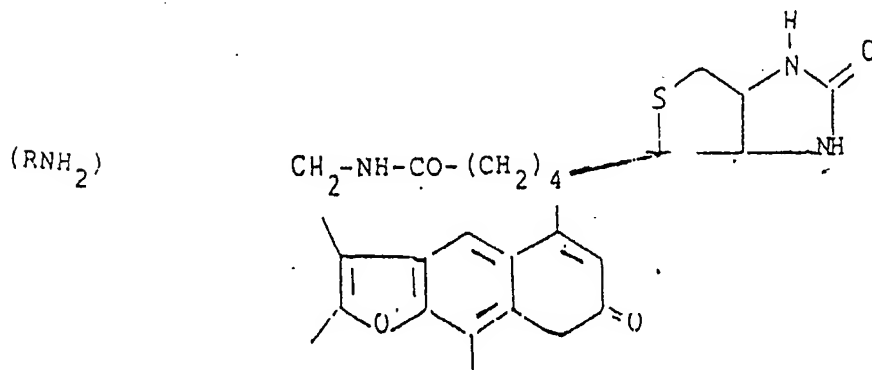
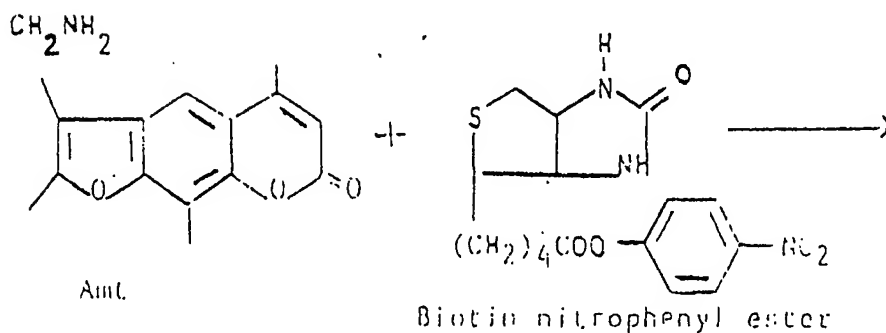
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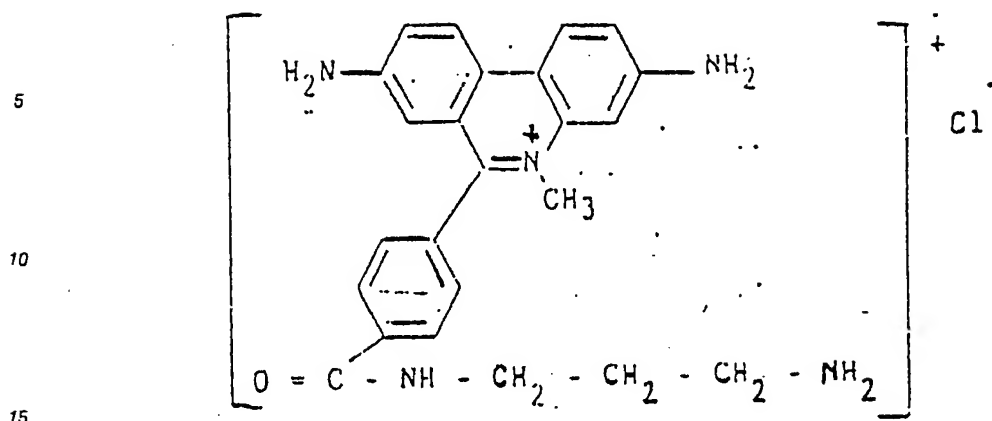
or



By way of example,



Other aminomethylangelicin, psoralen and phenanthridium derivatives can be similarly reacted, as can phenanthridium halides and derivatives thereof such as aminopropyl methidium chloride, i.e.,



(see Hertzberg et al, *J. Amer. Chem. Soc.*, **104**, 313 (1982)).

Alternatively, a bifunctional reagent such as dithiobis succinimidyl propionate or 1,4-butanediol diglycidyl ether can be used directly to couple the photochemically reactive molecule with the label where the reactants have alkyl amino residues, again in a known manner with regard to solvents, proportions and reaction conditions. Certain bifunctional reagents, possibly glutaraldehyde may not be suitable because, while they couple, they may modify nucleic acid and thus interfere with the assay. Routine precautions can be taken to prevent such difficulties.

The particular sequence used in making the labeled nucleic acid can be varied. Thus, for example, an amino-substituted psoralen can first be photochemically coupled with a nucleic acid, the product having pendant amino groups by which it can be coupled to the label, i.e., labeling can be carried out by photochemically reacting a DNA binding ligand with the nucleic acid in the test sample. Alternatively, the psoralen can first be coupled to a label such as an enzyme and then to the nucleic acid.

As described in pending EP-patent application No. 85 116 199.2, filed December 18, 1985, the present invention also encompasses a labeled nucleic acid comprising (a) a nucleic acid component, (b) a nucleic acid-binding ligand photochemically linked to the nucleic acid component, (c) a label and (d) a spacer chemically linking (b) and (c).

Advantageously, the spacer includes a chain of up to about 40 atoms, preferably about 2 to 20 atoms, selected from the group consisting of carbon, oxygen, nitrogen and sulfur.

Such spacer may be the polyfunctional radical of a member selected from the group consisting of peptide, hydrocarbon, polyalcohol, polyether, polyamine, polyimine and carbohydrate, e.g., -glycyl-glycyl-glycyl- or other oligopeptide, carbonyl dipeptides, and omega-amino-alkane-carbonyl radical such as -NH-(CH₂)₆-CO-, a spermine or spermidine radical, an alpha, omega-alkanediamine radical such as -NH-(CH₂)₆-NH or -HN-CH₂-CH₂-NH, or the like. Sugar, polyethylene oxide radicals, glyceryl, pentaerythritol, and like radicals can also serve as the spacers.

These spacers can be directly linked to the nucleic acid-binding ligand and/or the label or the linkages may include a divalent radical of a coupler such as dithiobis succinimidyl propionate, 1,4-butanediol diglycidyl ether, a diisocyanate, carbodiimide, glyoxal, glutaraldehyde, or the like.

The spacer can be incorporated at any stage of the process of making the probe.

a-b-d-c

defined hereinabove. Thus, the sequence can be any of the following:

a+b+d+c,

b+d+c+a,

d+c+b+a,

b+d+a+c, etc.

The conditions for the individual steps are well known in chemistry.

If the label is an enzyme, for example, the product will ultimately be placed on a suitable medium and the extent of catalysis will be determined. Thus, if the enzyme is a phosphatase, the medium could contain nitrophenyl phosphate and one would monitor the amount of nitrophenol generated by observing the color.

If the enzyme is a beta-galactosidase, the medium can contain o-nitro-phenyl-D-galacto-pyranoside which also will liberate nitrophenol.

The labeled nucleic acid of the present invention is applicable to all conventional hybridization assay formats, and in general to any format that is possible based on formation of a hybridization product or aggregate comprising the labelled nucleic acid. In particular, the unique labelled nucleic acid of the present invention can be used in solution and solid-phase hybridization formats, including, in the latter case, formats involving immobilization of either sample or probe nucleic acids and sandwich formats.

The nucleic acid probe will comprise at least one single-stranded base sequence substantially complementary to or homologous with the sequence to be detected. However, such base sequence need not be a single continuous polynucleotide segment, but can be comprised of two or more individual segments interrupted by nonhomologous sequences. These nonhomologous sequences can be linear or they can be self-complementary and form hairpin loops. In addition, the homologous region of the probe can be flanked at the 3' and 5' termini by nonhomologous sequences, such as those comprising the DNA or RNA or a vector into which the homologous sequence had been inserted for propagation. In either instance, the probe as presented as an analytical reagent will exhibit detectable hybridization at one or more points with sample nucleic acids of interest. Linear or circular single-stranded polynucleotides can be used as the probe element, with major or minor portions being duplexed with a complementary polynucleotide strand or strands, provided that the critical homologous segment or segments are in single-stranded form and available for hybridization with sample DNA or RNA. Useful probes include linear or circular probes wherein the homologous probe sequence is in essentially only single-stranded form (see particularly, Hu and Messing, Gene, 17:271 (1982)).

The nucleic acid probe of the present invention can be used in any conventional hybridization technique. As improvements are made and conceptually new formats are developed, such can be readily applied to the present probes. Conventional hybridization formats which are particularly useful include those wherein the sample nucleic acids or the polynucleotide probe is immobilized on a solid support (solid-phase hybridization) and those wherein the polynucleotide species are all in solution (solution hybridization).

In solid-phase hybridization formats, one of the polynucleotide species participating in hybridization is fixed in an appropriate manner in its single-stranded form to a solid support. Useful solid supports are well known in the art and include those which bind nucleic acids either covalently or noncovalently. Noncovalent supports which are generally understood to involve hydrophobic bonding include naturally occurring and synthetic polymeric materials, such as nitrocellulose, derivatized nylon and fluorinated polyhydrocarbons, in a variety of forms such as filters, beads or solid sheets. Covalent binding supports (in the form of filters, beads or solid sheets, just to mention a few) are also useful and comprise materials having chemically reactive groups or groups, such as dichlorotriazine, diazobenzylloxymethyl, and the like, which can be activated for binding to polynucleotides.

It is well known that noncovalent immobilization of an oligonucleotide is ineffective on a solid support, for example, on nitrocellulose paper. The present invention also describes novel methods of oligonucleotide immobilization. This is achieved by phosphorylation of an oligonucleotide by a polynucleotide kinase or by ligation of the 5'-phosphorylated oligonucleotide to produce multi-oligonucleotide molecules capable of immobilization. The conditions for kinase and ligation reaction have been described in standard text books, e.g., Molecular Cloning, T. Maniatis, E.F. Fritsch and J. Sambrook, Cold Spring Harbor Laboratory, (1982), pages 1-123.

A typical solid-phase hybridization technique begins with immobilization of sample nucleic acids onto the support in single-stranded form. This initial step essentially prevents reannealing of complementary strands from the sample and can be used as a means for concentrating sample material on the support for enhanced detectability. The polynucleotide probe is then contacted with the support and hybridization detected by measurement of the label as described herein. The solid support provides a convenient means for separating labelled probe which has hybridized to the sequence to be detected from that which has not hybridized.

Another method of interest is the sandwich hybridization technique wherein one of two mutually exclusive fragments of the homologous sequence of the probe is immobilized and the other is labelled. The presence of the polynucleotide sequence of interest results in dual hybridization to the immobilized and labelled probe segments. See Methods in Enzymology, 65:468 (1980) and Gene, 21:77-85 (1983) for further details.

For the present invention, the immobile phase of the hybridization system can be a series or matrix of spots of known kinds and/or dilutions of denatured DNA. This is most simply prepared by pipetting appropriate small volumes of native DNA onto a dry nitrocellulose or nylon sheet, floating the sheet on a sodium hydroxide solution to denature the DNA, rinsing the sheet in a neutralizing solution, then baking the sheet to fix the DNA. Before DNA:DNA hybridization, the sheet is usually treated with a solution that inhibits non-specific binding of added DNA during hybridization.

This invention involves the labeling of whole genomic DNA, whole nucleic acids present in cells, whole cell lysate, or unlysed whole cells. Once the labeled material is prepared, it can be used for the detection, i.e., the presence or absence of certain specific genomic sequences by specific nucleic acid hybridization assays.

5 One method according to the invention involves the separation of cells from a human sample or the human sample directly is treated by mixing with a photochemically reactive nucleic acid binding intercalating ligand. The mixture is incubated depending on the type of the sample. If the sample is lysed cells or nucleic acids, it is incubated for a period between a few seconds to five minutes and when whole cells or partially lysed cells are used, incubation between two minutes to two hours is employed. After the mixing
10 and incubation, the whole sample mixture is irradiated at a particular wavelength for the covalent interaction between the photochemically reactive DNA binding ligand and the test sample. Then this labeled material is hybridized under specific hybridization conditions with a specific probe.

After the hybridization, the unreacted unhybridized labeled test sample is removed by washing. After the washing, the hybrid is detected through the label carried by the test sample, which is specifically
15 hybridized with a specific probe.

The present invention is surprising since in a human genomic sample the amount of a single copy gene is very low, for example, if a restriction fragment of one thousand base pair is the region of hybridization, the probability of such sequence in the whole human genomic sample is one in a million. This conclusion has been derived by assuming from the literature that a human genomic sample has 3×10^9 base pairs and
20 1000 base pairs will be 1/3,000,000 of that number. Automatically, in a sample of human DNA containing approximately five to ten micrograms of nucleic acids, only 5 to 10 picogram of the corresponding sequences is available and labeling the vast majority of the non-specific DNA should produce more background than the true signal. But after the reaction, it is surprising to observe that the results are not only specific, but also of unexpected higher sensitivity.

Without wishing to be bound by any particular theory of operability, the reason for the unexpected sensitivity may be due to the formation of a network of non-specific nucleic acid hybrids bound to the specific hybrid, thus amplifying the amount of the signal. As has been shown in a typical example, a 19 nucleotide long specific sequence containing plasmid is immobilized and hybridized with 5 microgram equivalent of a test sample which is labeled photochemically and one detects very easily the signal resulted
30 from such hybrid. This could not have been accomplished by any other technique because of the problems associated with the labeling method.

The present invention relates to a novel hybridization technique where probes are immobilized and an eukaryotic nucleic acid sample is labeled and hybridized with immobilized unlabeled probe. A surprising characteristic of the invention is the ability to detect single or multiple copy gene defects by labeling the
35 test sample. Since there is no requirement for an excess of labeled hybridizing sequence, the present method is more specific. In the present invention, simultaneous detection of different gene defects can be easily carried out by immobilizing specific probes.

For example, using the present invention, one can immobilize oligonucleotide probes specific for genetic defects related to sickle cell anemia and probes for alpha-thalassemias on a sheet of nitrocellulose
40 paper, label the test sample and hybridize the labeled test sample with the immobilized probes. It is surprising that partially purified or unpurified nucleic acid samples (cell lysate or whole cell) can be photochemically labeled with sensitive molecules without affecting the specific hybridizability.

The present invention is also directed to detecting eukaryotes (protists) in samples from higher organisms, such as animals or humans.

45 Eukaryotes include algae, protozoa, fungi and slime molds.

The term "algae" refers in general to chlorophyll-containing protists, descriptions of which are found in G.M. Smith, Cryptogamic Botany, 2nd ed. Vol. 1, Algae and Fungi, McGraw-Hill, (1955).

Eukaryotic sequences according to the present invention includes all disease sequences except for bacteria and viruses. Accordingly, genetic diseases, for example, would also be embraced by the present
50 invention. Non-limiting examples of such genetic diseases are as follows:

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	<u>Area Affected</u>	<u>Diseases</u>
10	Metabolism	Acute intermittent porphyria Variegate porphyria alpha ₁ -antitrypsin deficiency
15		Cystic fibrosis Phenylketonuria Tay-Sachs disease
20		Mucopolysaccharidosis I Mucopolysaccharidosis II Galactosaemia
25		Homocystinuria Cystinuria Metachromic leucodystrophy
30	Nervous System	Huntington's chorea Neurofibromatosis Myotonic dystrophy Tuberous sclerosis Neurogenic muscular atrophies
35		
40	Blood	Sickle-cell anaemia Beta-thalassaemia Congenital spherocytosis Haemophilia A
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	Bowel	Polyposis coli
50	Kidney	Polycystic disease

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	Eyes	Dominant blindness Retinoblastoma
5	Ears	Dominant early childhood deafness Dominant otosclerosis
10	Circulation	Monogenic hypercholesterolaemia
	Blood	Congenital spherocytosis
15	Teeth	Dentinogenesis imperfecta Amelogenesis imperfecta
20	Skeleton	Diaphysial aclasia Thanatophoric dwarfism Osteogenesis imperfecta Marfan syndrome Achondroplasia Ehlers-Danlos syndrome Osteopetrosis tarda Cleft lip/palate
30	Skin	Ichthyosis
35	Locomotor	Muscular dystrophy

A nucleic acid probe in accordance with the present invention is a sequence which can determine the sequence of a test sample. The probes are usually DNA, RNA, mixed copolymers of ribo- and deoxyribonucleic acids, oligonucleotides containing ribonucleotides or deoxyribonucleotide residues or their modified forms. The sequence of such a probe should be complementary to the test sequence. The extent of complementary properties will determine the stability of the double helix formed after hybridization. The probe can also have covalently linked non-complementary nucleic acids. They can serve as the sites of the labeling reaction.

The nucleic acid is preferably labeled by means of photochemistry, employing a photoreactive DNA-binding furocoumarin or a phenanthridine compound to link the nucleic acid to a label which can be "read" or assayed in conventional manner, including fluorescence detection.

One use of the present invention is the identification of bacterial species in biological fluids. In one application, samples of urine from subjects having or suspected of having urinary tract infections can provide material for the preparation of labelled DNA(s) or RNAs, while a solid support strip, e.g., made of nitrocellulose or nylon, can contain individual dots or spots of known amounts of denatured purified DNA from each of the several bacteria likely to be responsible for infection.

The format of labeled unknown and unlabeled probes, which is the converse of standard schemes, allows one to identify among a number of possibilities the species of organism in a sample with only a single labeling. It also allows simultaneous determination of the presence of more than one distinguishable bacterial species in a sample (assuming no DNA in a mixture is discriminated against in the labeling procedure). However, it does not allow in a simple way, better than an estimate of the amount of DNA (and, therefore, the concentration of bacteria) in a mixed sample. For such quantitation, sample DNA is immobilized in a series of dilution spots along with spots of standard DNA, and probe DNAs are labeled.

A urinary tract infection is almost always due to monoclonal growth of one of the following half dozen kinds of microorganism: Escherichia coli (60-90% of UTI), Proteus spp. (5-20% of UTI), Klebsiella spp (3-10% of UTI), Staphylococcus spp. (4-20% of UTI), Streptococcus spp. (2-5% of UTI). Pseudomonas and some other gram negative rods together account for a low percentage of UTI. A common contaminant of urine samples that is a marker of improper sample collection is Lactobacillus.

The concentration of bacteria in a urine sample that defines an infection is about 10^5 per milliliter.

The format for an unlabeled probe hybridization system applicable to urinary tract infections is to have a matrix of DNAs from the above list of species, denatured and immobilized on a support such as nitrocellulose, and in a range of amounts appropriate for concentrations of bacterial DNAs that can be expected in samples of labelled unknown.

Standard hybridization with biotinylated whole genome DNA probes takes place in 5-10 ml, at a probe concentration of about 0.1 $\mu\text{g/ml}$; hybridization of probe to a spot containing about 10 ng denatured DNA is readily detectable. There is about 5 fg of DNA per bacterial cell, so that for a sample to contain 1 μg of labelled DNA, it is necessary to collect 2×10^8 bacteria. If an infection produces urine having approximately 10^5 bacteria/ml, then bacterial DNA to be labeled from a sample is concentrated from 2000 ml. If more than 10 ng unlabeled probe DNA is immobilized in a dot, for example, 100 ng or 1 μg , or if the hybridization volume is reduced, then the volume of urine required for the preparation of labeled unknown is approximately a few tenths of a ml.

A strip of dots containing immobilized, denatured, unlabelled probe DNAs could have the following configuration:

	1 μg	10 ng	100 pg
Escherichia	○	○	○
Proteus	○	○	○
Klebsiella	○	○	○
Staphylococcus	○	○	○
Streptococcus	○	○	○
Pseudomonas	○	○	○
Lactobacillus	○	○	○

This procedure involves the labeling of DNA or RNA in a crude cell lysate. Ideally, preparation of labeled sample DNA or RNA will accommodate the following points:

- (1) bacteria will be concentrated from a fluid sample by centrifugation or filtration;
- (2) bacteria will be lysed under conditions sufficient to release nucleic acids from the most refractory of the organisms of interest;
- (3) the labeling protocol will not require purification of labeled nucleic acids from unincorporated precursors, nor the purification of nucleic acids prior to labeling;
- (4) the labeling protocol will be sufficiently specific for DNA and/or RNA that proteins, lipids and polysaccharides in the preparation will not interfere with hybridization nor read-out.

In the present invention, there is provided a method for efficiently and rapidly lysing whole cells, including Gram positive bacteria. The method involves contacting cells, e.g., whole cells, with an alkali, e.g., sodium or potassium hydroxide solution in a concentration of 0.1 to 1.6 Normal.

The important features of the present lysis protocol are its relative simplicity and speed. It employs a common chemical that requires no special storage conditions and it lyses even Gram positive organisms with high efficiency, while preserving the properties of the DNA that are important for subsequent steps in the photochemical labeling process.

For the present invention, the immobile phase of the hybridization system can be a series or matrix of spots of known kinds and/or dilutions of denatured DNA. This is most simply prepared by pipetting appropriate small volumes of native DNA or oligonucleotides onto a dry nitrocellulose or nylon sheet, floating the sheet on a sodium hydroxide solution to denature the DNA, rinsing the sheet in a neutralizing solution, then baking the sheet to fix the DNA. Before DNA:DNA hybridization, the sheet is usually treated with a solution that inhibits non specific binding of added DNA during hybridization.

The invention will be further described in the following non-limiting examples wherein parts are by weight unless otherwise expressed.

Examples

Example 1: Preparation of Labelling Compound

The preparation of the labeling compound required 1-amino-17-N-(Biotinylamido)-3,6,9,12,15 penta-oxaheptadecane. This compound was prepared in the following four steps:

- (a) 3,6,9,12,15 penta-oxaheptadecane 1,17-diol ditosylate was synthesized.
- (b) 1,17-dipthalimido derivative of 3,6,9,12,15 penta-oxaheptadecane was prepared.
- (c) 1,17-diamino derivative of 3,6,9,12,15 penta-oxaheptadecane was prepared.
- (d) 1-amino, 17-biotinylamido derivative of 3,6,9,12,15 penta-oxaheptadecane was prepared.

Example 1(a): Preparation of 3,6,9,12,15-Penta-oxaheptadecane-1,17-diol Ditosylate

To a stirred solution containing 50 g of hexaethylene glycol (0.177 mol) and 64 ml of triethylamine - (39.36 g, 0.389 mol) in 400 ml of CH_2Cl_2 at 0°C was added dropwise a solution containing 73.91 g of p-toluenesulfonyl chloride (0.389 mol) in 400 ml of CH_2Cl_2 over a 2.5 hour period. The reaction mixture was then stirred for one hour at 0°C and then heated to ambient temperature for 44 hours. The mixture was then filtered and the filtrate was concentrated in vacuo. The resulting heterogeneous residue was suspended in 500 ml of ethyl acetate and filtered. The filtrate was then concentrated in vacuo to a yellow oil which was triturated eight times with 250 ml portions of warm hexane to remove unreacted p-toluenesulfonyl chloride. The resulting oil was then concentrated under high vacuum to yield 108.12 g of a yellow oil (quantitative yield).

Analysis: Calculated for $\text{C}_{26}\text{H}_{48}\text{O}_{11}\text{S}_2$

Calc.: C, 52.87; H, 6.48.

found: C, 52.56; H, 6.39.

PMR: (60 MHz, CDCl_3) δ : 2.45 (s, 6H); 3.4-3.8 (m, 20H); 4.2 (m, 4H); 7.8 (AB quartet, $J = 8\text{Hz}$, 8H).

IR: (neat) cm^{-1} : 2870, 1610, 1360, 1185, 1105, 1020, 930, 830, 785, 670.

Example 1(b): Preparation of 1,17-Dipthalimido-3,6,9,12,15-penta-oxaheptadecane

A stirred suspension containing 108 g of 3,6,9,12,15-penta-oxaheptadecane-1,17-diol ditosylate (0.183 mol), 74.57 g of potassium phthalimide (0.403 mol), and 700 ml of dimethylacetamide was heated at $160-170^\circ\text{C}$ for 2 hours and was then cooled to room temperature. The precipitate was filtered and washed with water and acetone to yield 53.05 g of product as a white powder which was dried at 55°C (0.1 mm). mp $124-126^\circ\text{C}$.

A second crop of product was obtained from the dimethylacetamide filtrate by evaporation in vacuo and the resulting precipitate with was successively washed ethyl acetate, water, and acetone. The resulting white powder was dried at 55°C (0.1 mm) to yield an additional 9.7 g of product. mp $124.5-126.5^\circ\text{C}$. The combined yield of product was 62.82 g (68% yield).

Analysis: (For first crop)

Calculated for $\text{C}_{28}\text{H}_{32}\text{N}_2\text{O}_9 \cdot 1/2\text{H}_2\text{O}$

Calc.: C, 61.19; H, 6.05; N, 5.09.

found: C, 61.08; H, 6.15; N, 5.05.

(For second crop)

Calculated for $C_{28}H_{32}N_2O_5$

Calc.: C, 62.21; H, 5.97; N, 5.18.

found: C, 61.78; H, 6.15; N, 5.13.

PMR: (60 MHz, $dms\text{-}d_6$) δ : 3.5 (s, 8H); 3.6 (s, 8H); 3.8 (bt, $J=3\text{Hz}$, 8H); 8.1 (s, 8H).

IR: (KBr) cm^{-1} : 2890, 1785, 1730, 1400, 1100, 735.

Example 1(c): Preparation of 1,17-Diamino-3,6,9,12,15-Pentaoxaheptadecane

10

A solution containing 60 g of 1,17-diphthalimido-3,6,9,12,15-pentaoxaheptadecane (0.118 mol), 14.8 g of hydrazine hydrate (0.296 mol), and 500 ml of ethanol were heated with mechanical stirring in a 100°C oil bath for three hours. The mixture was then cooled and filtered. The resultant filter cake was washed four times with 300 ml portions of ethanol. The combined filtrates were concentrated to yield 32.35 g of a yellow opaque glassy oil. The evaporative distillation at 150-200°C (0.01 mm) gave 22.82 g of a light yellow oil - (69% yield). lit. b.p. 175-177°C (0.07 mm).

15

PMR: (60 MHz, CDCl_3) δ : 1.77 (s, 4H, NH_2); 2.85 (t, $J=5\text{Hz}$, 4H); 3.53 (t, $J=5\text{Hz}$, 4H); 3.67 (m, 16H).

IR: (CHCl_3) cm^{-1} : 3640, 3360, 2860, 1640, 1585, 1460, 1350, 1250, 1100, 945, 920, 870.

Mass Spectrum: (EI) m/e = 281.2 (0.1%, $M+1$).

20

(FAB) m/e = 281.2 (100%, $M+1$).

Analysis: For $C_{12}H_{28}N_2O_5 \cdot 1/2 \text{H}_2\text{O}$

Calc.: C, 49.80, H, 10.10; N, 9.68.

found: C, 50.36, H, 9.58; N, 9.38.

Literature Reference: W. Kern, S. Iwabachi, H. Sato and V. Bohmer, *Makrol. Chem.*, **180**, 2539 (1979).

25

Example 1(d): Preparation of 1-Amino-17-N-(Biotinylamido)-3,6,9,12,15-pentaoxaheptadecane

A solution containing 7.2 g of 1,17-diamino-3,6,9,12,15-pentaoxaheptadecane (25 mmol) in 75 ml of DMF under an argon atmosphere was treated with 3.41 g of N-succinimidyl biotin (10 mmol) added in portions over 1.0 hour. The resulting solution was stirred for four hours at ambient temperature. TLC (SiO_2 , 70:10:1 CHCl_3 - CH_3OH -conc. NH_4OH) visualized by dimethylaminocinnamaldehyde spray reagent showed excellent conversion to a new product ($R_f=0.18$). The reaction mixture was divided in half and each half was absorbed onto SiO_2 and flash-chromatographed on 500 g of SiO_2 -60 (230-400 mesh) using a 70:10:1 CHCl_3 - CH_3OH -conc. NH_4OH solvent mixture. Fractions containing the product were pooled and concentrated to a yield 2.42 g of a gelatinous, waxy solid. The product was precipitated as a solid from isopropanol-ether, washed with hexane, and dried at 55°C (0.1 mm) to give 1.761 g of a white powder (35% yield).

35

Analysis: Calculated for $C_{22}H_{47}N_4O_7S \cdot 3/2 \text{H}_2\text{O}$:

C, 49.51; H, 8.50; N, 10.49.

40

found: C, 49.59; H, 8.13; N, 10.39.

PMR: (90 MHz, $dms\text{-}d_6$) δ : 1.1-1.7 (m, 6H); 2.05 (t, $J=7\text{Hz}$, 2H);

2.62 (t, $J=4\text{Hz}$, 1H); 2.74 (t, $J=4\text{Hz}$, 1H); 3.0-3.4 (m, 14H).

3.50 (s, 14H); 4.14 (m, 1H); 4.30 (m, 1H); 6.35 (d, $J=4\text{Hz}$, 1H); 7.80 (m, 1H).

45

CMR: (22.5 MHz, $dms\text{-}d_6$) δ : 25.2, 28.0, 28.2, 35.1, 40.6, 55.3, 59.2, 61.1, 69.6, 69.8, 71.2, 162.7, 172.1.

IR: (KBr) cm^{-1} : 2900, 2850, 1690, 1640, 1580, 1540, 1450, 1100.

Mass Spectrum (FAB) m/e : 507.3 ($M+1$, 56%)

Example 2: Preparation of 4'-Biotinyl-PEG-4,5'-dimethylangelicin

A solution of 203 mg of 1-amino-17-N-(biotinylamido)-3,6,9,12,15-pentaoxaheptadecane (0.4 mmol) in 1 ml of DMF under an argon atmosphere was treated with 78 mg of N,N-carbonyldimidazole (0.48 mmol). The resulting mixture was stirred for four hours and was then treated with 55 mg of 4'-aminomethyl-4,5'-dimethylangelicin hydrochloride (0.2 mmol), 140 μl of diisopropylethylamine, and 100 μl of DMF. The resulting

55

mixture was stirred overnight at 50°C. The mixture was then evaporated onto SiO₂ *in vacuo* and the resultant impregnated solid flash was chromatographed on 60 g of SiO₂ (230-400 mesh) eluted with 1.5 liters of 7% CH₃-CHCl₃, followed by 1 liter of 10% CH₃OH-CHCl₃. Fractions containing the product were pooled and concentrated to yield 72 mg of a glassy solid (47% yield).

5 PMR: (90 MHz, dmsd-d₆): δ 1.1-1.8 (m, 6H); 2.04 (bt, J=7Hz, 2H); 2.5 (s, 6H); 2.56 (m, 1H); 2.74 (bd, J=4Hz, 1H); 2.8-3.4 (m, 14H); 3.40 (m, 14H); 4.14 (m, 1H); 4.25 (m, 1H); 4.40 (bd, J=6Hz, 2H); 6.5 (m, 1H); 6.35 (s, 1H); 7.02 (s, 1H); 7.45 (d, J=8Hz, 1H); 7.62 (d, J=8Hz, 1H); 7.80 (m, 1H).

CMR: (22.5 MHz, dmsd-d₆) δ : 11.9, 18.9, 25.3, 28.2, 28.3, 33.4, 35.2, 55.4, 59.2, 61.0, 69.2, 69.6, 69.8, 70.0, 89.0, 107.8, 112.0, 113.1, 114.3, 120.6, 121.6, 153.6, 154.4, 155.6, 157.9, 159.5, 162.7, 172.1.

10 Literature Reference: F. Dall'Acqua, D. Vedaldi, S. Caffieri, A. Guiotto, P. Rodighiero, F. Baccichetti, F. Carlassare and F. Bordin, J. Med. Chem., **24**, 178 (1981).

Example 3: Colorimetric or Chemiluminescent Detection of the Nucleic Acid Hybrids

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Example 3(a): Colorimetric Detection

Colorimetric detection of the biotinylated hybrids is carried out following the procedure and kit developed by Bethesda Research Laboratories (BRL), Gaithersburg, Maryland 20877, U.S.A. The procedure is described in detail in a manual supplied with a kit by BRL, entitled "Products for Nucleic Acid Detection", "DNA Detection System Instruction Manual", Catalogue No. 8239SA.

20

Example 3(b): Chemiluminescent Detection

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Chemiluminescent detection of the biotinylated hybrids is identical to the above method: the filters with the hybrids are saturated with BSA (bovine serum albumin) by immersing the paper in 3% BSA at 42°C for 20 minutes. Excess BSA is removed by taking the paper out of the container, and blotting it between two pieces of filter paper. The paper is then incubated in a solution containing Streptavidin (0.25 mg/ml, 3.0 ml total volume), for 20 minutes at room temperature. It is then washed three times with a buffer containing 0.1 M Tris-HCl, pH 7.5, 0.1 M NaCl, 2mM MgCl₂, 0.05% "TRITON X-100". Next the filter is incubated with biotinylated horseradish peroxidase (0.10 mg/ml) for 15 minutes at room temperature. This is followed by three washings with 0.1 M Tris-HCl, pH 7.5, 0.1 M NaCl, 2 mM MgCl₂, and 0.05% Triton X-100, and one washing with 10 mM Tris (pH 8.0) buffer. Chemiluminescent activation is conducted in two ways. (1) Spots are punched out and the discs containing the DNA are placed in a microtiter plate with wells that are painted black on the sides. After the punched paper circles are placed in the microtiter place wells, 0.8 ml buffer containing 40 mM Tris and 40 mM ammonium acetate (pH 8.1) is added to each well. Then 10 μ l of 1:1 mixture of 39 mM Luminol (in DMF) and 30 mM H₂O₂ (in water) is added. Light emission is recorded on a "POLAROID" instant film by exposing it directly in the film holder. Alternatively (2), the paper is soaked in a solution containing 1:1 mixture of 0.5 mM Luminol and H₂O₂ and wrapped with a transparent "SARAN WRAP". The light emission is recorded on a "POLAROID" film as above.

35

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Example 4: General Method of Labeling the Test Sample Nucleic Acids

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High molecular weight DNA from a patient's sample is isolated by a method described in U.S.P. 4,395,486 (Wilson et al), the entire contents of which are incorporated by reference herein. The nucleic acid is dissolved in 10 mM borate buffer (pH 8.0) to a final concentration of approximately 20 μ g/ml. To the nucleic acid solution "angelicin-peg-biotin" in aqueous solution is added to a final concentration of 10 μ g/ml. The mixture is then irradiated at long wavelength irradiation for about 60 minutes using a black ray UVL 56 lamp. The product is ready for hybridization without purification. However, the product can be purified by dialysis or alcohol precipitation (U.S.P. 4,395,486) as is usually followed for nucleic acids.

50

Instead of nucleic acids, whole cell lysate can also be labeled following an identical procedure. The lysis is conducted by boiling the cells with 0.1 N sodium hydroxide, followed by neutralization with hydrochloric acid.

55

When whole cells are used, the mixture of "PEG-ang-bio" and cells are incubated for at least 60 minutes prior to irradiation for efficient transport of the ligands. Many different variations of the above described methods can be adopted for labeling.

5

Example 5:

Alpha-thalassemia is associated with gene deletion. The detection of gene deletion by hybridization in a dot/slot blot format requires that the total amount of sample and its hybridizability are accurately known. Since the beta-globin gene is a single copy gene, simultaneous hybridization of a sample with beta-globin and alpha-globin and their relative amounts will indicate the amount of alpha-globin with the sample.

The format and hybridization conditions are the same as Rubin and Kan, supra, except probes, not test DNA, is immobilized. Hybridization conditions are also similar. The detection is done by using the BRL kit described supra following BRL's specifications.

15 The hybridization detection process are conducted in three steps as follows:

Step 1: Immobilization of the Probes

20 As described in Rubin and Kan, supra, 1.5 kb PstI fragment containing alpha, globin gene is used as a probe for alpha-thalassemia and for the beta-globin gene a 737 base pair probe produced by the digestion of pBR beta Pst (4.4 kb) is used. The beta-globin gene probe has been described in U.S.P. 4,395,486 (column 4). For the detection of gene deletion related to alpha-thalassemia, the amount of starting nucleic acid, hybridization efficiency and control samples are needed. The present invention avoids these problems by simultaneous hybridization with a single copy essential gene (e.g., beta-globin gene) when similar amounts of probes are immobilized side by side, labeled sample is hybridized, relative strength of signal intensity is a measure of relative amount of gene dosage present in the sample.

The probes (0.5, 1, 3 and 5 µg per 100 µl) are suspended in 10 mM tris HCl (pH 7) buffer, denatured with 20 µl 3 M sodium hydroxide, at 100°C, for 5 minutes, an equivalent volume of 2 M ammonium acetate, pH 5.0 is added to neutralize the solution, immediately after neutralization the probes for beta-and alpha-globin genes are applied in parallel rows to nitrocellulose filter paper under vacuum in a slot blot manifold, purchased from Schleicher and Schuell, (Keen, New Hampshire, U.S.A.). The filter is then dried in vacuum at 80°C for 60 minutes. It is then prehybridized for 4 hours in a mixture containing 50 mM sodium phosphate (pH 7) 45 mM sodium citrate, 450 mM sodium chloride, 50% (v/v) formamide, 0.2% each (w/v) of polyvinyl pyrrolidone, "FICOLL 400" and bovine serum albumin and 0.2 mg/ml alkali boiled salmon sperm DNA and 0.15 mg/ml yeast RNA.

Step 2: Labeling of the Test Sample

40

This was described above.

Step 3: Hybridization

45

The nitrocellulose strip containing the immobilized probes are hybridized with the labeled test sample in plastic bags (e.g., "SEAL-A-MEAL", "SEAL and SAVE", etc.). Hybridization solution is the same as prehybridization solution plus 10% dextran sulphate. Hybridization is done at 42°C for 16 hours. After hybridization detection of biotin is conducted with a kit and procedure supplied by Bethesda Research Laboratory, Maryland, U.S.A., (catalogue No. 8239SA). Results of relative intensity of alpha-and beta-regions are used to estimate the extent of deletion of alpha-globin genes:

No signal on the alpha-globin side: all 4 alpha-globin genes missing.

Signal on the alpha-globin side is half as strong as on the corresponding beta-side: 3 alpha-globin genes missing.

55 Signals on alpha and beta side equivalent: 2 alpha-globin genes missing.

Signals on alpha side is stronger than the corresponding beta side (2 alpha = 3 beta); 1 alpha-globin gene missing.

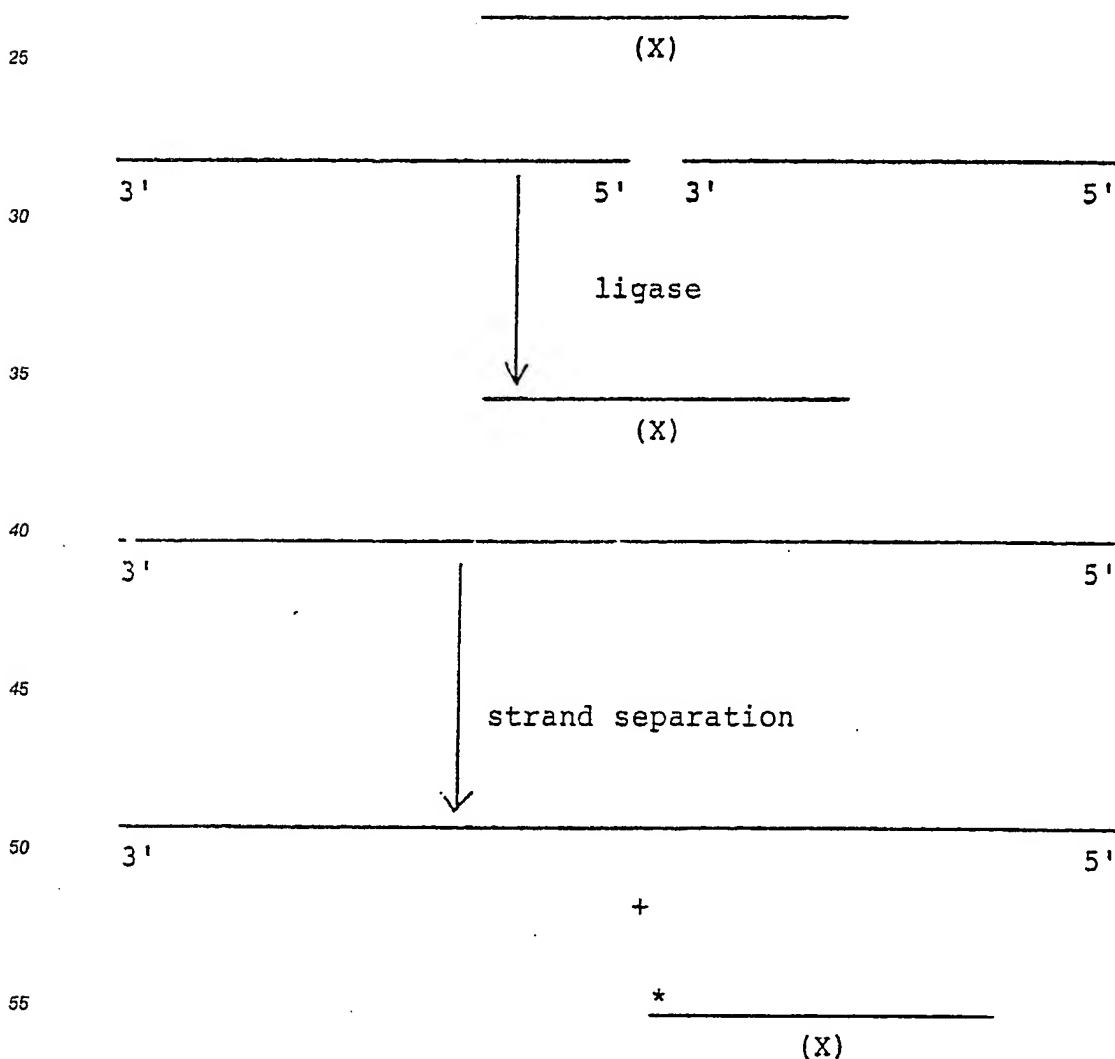
5 Example 6: Immobilization of an Oligonucleotide Sequence Specific for Hemoglobin Mutation

It is known that an oligonucleotide cannot be easily immobilized onto nitrocellulose paper by a simple adsorption process. The present invention encompasses three different methods to incorporate an oligonucleotide sequence into a larger molecule capable of adsorption.

10 Method 1: Two oligonucleotides, one a 43mer and the other a 16-mer, have been chemically synthesized in an automated synthesizer (Applied Biosystem 380B) by the phosphoramidite-method and phosphorylated at the 5' end by a T4-polynucleotide kinase mediated process according to Maniatis et al, Molecular Cloning, page 122. These oligonucleotides contain a segment of a 19 nucleotide long sequence specific for the detection of the mutation associated with sickle cell anemia.

15 43mer A & S (A = normal globin gene; S = sickle globin gene) were kinased according to Maniatis et al, Molecular Cloning, page 122, in two separate reactions, namely, one with ^{32}P -ATP and one with no radioactive label. 0.4 μg ^{32}P -43mer and 0.6 mg cold 43mer were mixed and purified on a spun column (G-25med in TE (Tris EDTA buffer)) to a final volume of 40 μl . Two dilutions were spotted on S & S (Schleicher & Schuell) nitrocellulose and nytran (nylon) membranes at 50 and 0.5 ng.

20 Method 2: The phosphorylated oligonucleotide products of method 1 were further elongated by making multimers of sequences by a ligase mediated process. The principle is described as follows:



The product being of a higher molecular weight than an oligonucleotide it should be immobilizable by adsorption on to a nitrocellulose paper.

Aqueous solutions containing 4 µg of ³²P43mer and 3.7 µg 16mer linker (X) were mixed and dried under vacuum. 6 mg of cold kinased 43mer was added and the sample was heated to 55°C and cooled slowly to 0°C to anneal. Ligation was carried out in 20 µl total reaction volume with 800 units of ligase (Pharmacia) at 15°C for 4 hours. 1 mg (2 µl) was purified on a spun column (G-25med in TE) to a final volume of 40 µl. Two dilutions were spotted on nitrocellulose nylon membranes at 50 and 0.5 ng.

Method 3: The same as method 2, but ligation was not conducted. Instead of ligation, cross linking was conducted with an intercalator to keep the double stranded regions intact. Hence, the cross linked molecule will have several oligonucleotide sequences covalently linked to each other.

2 µg of ³²P43mer (for sequence P-50) was added to 2.9 mg of a 16mer (for sequence P-50) linker and purified on a spun column (G25med in TE) to a final volume of 40 µl. 6 mg of kinased 43mer was added and the samples were heated to 55°C and cooled slowly to 0°C to anneal. 25 µl of intercalation compound aminomethyltrioxsalen was added and the sample was irradiated for 30 minutes on ice in 500 µl total 10 mM borate buffer pH 8.2 with a long wave UV lamp model (UVL-21, λ = 366 nm).

The probes modified by all three methods were then immobilized on to nitrocellulose and nylon paper and hybridized with labelled oligonucleotides. The results indicate that the sequence are immobilizable and hybridization fidelity remains intact.

Two dilutions of the products of methods 1 to 3 were spotted on nitrocellulose and nylon membranes at 50 and 0.5 ngs.

Whole filters were baked for 30 minutes in 80°C vacuum oven and prehybridized in blotto (5% nonfat dry milk, 6XSSC, 20 mM Na-pyrophosphate) for 30 minutes in 50°C oven.

Hybridization was carried out with primer extended 19'A & 19S' probes at 50°C for one hour (3 strips/probe).

Filters were stringently washed for 15 minutes at room temperature in 6XSSC with slight agitation and 2 x 10 minutes at 57°C.

Air-dried filters were placed on Whatman paper and autoradiographed at -70°C overnight.

The results presented in Fig. 1 surprisingly indicate specific hybridization are obtained by immobilizing oligonucleotide probes.

Example 7: Hybridization with labeled genomic DNA for Non Radioactive Detection

Human normal genomic (XX) DNA was photolabeled with "biotin-PEG-angelicin" (BPA) in 10 mM borate buffer pH 8.2 at a weight ratio of 0.3 to 1 (BPA:DNA) for 15 minutes on ice with a long wave UV lamp model UVL-21, λ = 366 nm. No purification is necessary.

Target DNA oligonucleotides were directly immobilized on S & S nitrocellulose in 1 µl aliquots at the following concentration, and then baked in an 80°C vacuum oven for 30 minutes. The amounts of the different immobilized probes are as follows:

43-mer	(A)	- Kinased (method 1)	200 ng
43-mer	(A)		200 ng
43-mer	(S)	- Kinased (method 1)	200 ng
43-mer	(S)		200 ng
M1319Ass			50 ng
M1319Sss			50 ng
M13737Ass			50 ng
BRL Commercially biotinylated DNA			200 pg
pUC19			50 ng

43-merA:

5' CTGCT,AATCTTAAGGAT,AAT,CTCCTGAGGAGAAGTCT GCT,AATCTTAA5,GGAT,AAT,CT 3'

• = T for 43-mer S

16-mer (Common to both A and S)

3' -TTAGAATTCCTAAATT-5'

5 Filters were prehybridized in blotto (5% nonfat dry milk, 6XSSC, 20 mM Na-pyrophosphate) for 30 minutes in a 45°C H₂O bath.

All 4 strips were hybridized in 2 mls solution containing 2 µg labeled XX DNA containing normal beta-globin gene (hybridization solution was blotto with 10% PEG) for 2 hours in 45°C in a H₂O bath.

A stringency wash was carried out as follows:

10 1 x 20' at room temperature in 6XSSC

2 x 20' at temperatures indicated in Fig. 2 with very little agitation.

50 ml centrifuge tubes were used for elevated temperature washes. Results are shown in Fig. 2.

15 Detection of biotin in the hybrid was carried out according to the Bethesda Research Laboratory, Bethesda, Maryland, U.S.A., manual using their kit for biotin detection. The results indicated specific hybridization.

Example 8: Immobilization of Whole Genomic DNA As Probes

20 Tens of milligram to gram amounts of DNA were prepared in the following manner from bacterial cells harvested from fermentor cultures. Bacteria were collected by centrifugation from 10 liter nutrient broth cultures grown in a New Brunswick Scientific Microferm Fermentor. Generally, cells in concentrated suspension were lysed by exposure to an ionic detergent such as SDS (Na dodecyl sulfate), then nucleic acids were purified from proteins and lipids by extraction with phenol and/or chloroform (J. Marmur, J. Mol. Biol., 3, 208-218, 1961). RNA was removed from the nucleic acids preparation by treatment of the DNA solution with 0.2 mg/ml ribonuclease at 37°C, then DNA was precipitated from solution by the addition of two volumes of ethanol. Bacterial DNA redissolved from the precipitate in a low salt buffer such as TE (10 mM Tris-HCl, pH 7.5, 1mM Na₂ EDTA) was characterized with respect to purity concentration and molecular size, then approximately 1 microgram aliquots were denatured and immobilized as spots on nitrocellulose or nylon membranes for hybridization (Kafatos et al., Nucleic Acids, Res. 7, 1541-1552, (1979)). Denaturation was accomplished by exposure of the DNA with approximately 0.1 N NaOH. After denaturation the solution was neutralized, then the membrane was rinsed in NaCl/Tris-HCl, pH 7.5, and dried.

Example 9: Processing of a Test Sample for Cellular DNA Labeling

35 Samples of urine, for example (although the following can equally apply to suspensions of material from gonorrhea-suspect swabs, from meningitis-suspect cerebrospinal fluid, from contamination-suspect water samples, etc.), are centrifuged or filtered to wash and concentrate any bacteria in the sample. The bacteria are then lysed by exposure to either (i) 2 mg/ml lysozyme or lysostaphin then exposure to approximately 90°C heat, (ii) 0.1 to 1.6 N NaOH, or (iii) 1% Na dodecyl sulfate. After (ii) NaOH, the cell lysate solution is neutralized before labelling; after (iii) detergent lysis, DNA labelling is preceded by removal of the SDS with 0.5 M K acetate on ice. Angelicin should be able to permeate intact cells so that DNA labeling can be accomplished before cell lysis. This in situ labeling simplifies the extraction procedure, as alkaline or detergent lysates can be incorporated directly into a hybridization solution.

45 Prior to hybridization, the labeled sample is denatured, and it should also preferably be reduced to short single stranded lengths to facilitate specific annealing with the appropriate unlabeled probe DNA. Methods of denaturation are known in the art. These methods include treatment with sodium hydroxide, organic solvent, heating, acid treatment and combinations thereof. Fragmentation can be accomplished in a control way by heating the DNA to approximately 80°C in NaOH for a determined length of time, and this, of course, also denatures the DNA.

Example 10: Labeling of the Products of Example 9

55 (i) A test sample of about 10ml urine will contain 10⁴ or more infectious agents. After separation by centrifugation and washing, the pretreated cell lysate (step 2) was resuspended in 0.2 ml 10 mM sodium borate buffer (pH approximately 8). To this suspension, 10 µg of photolabelling reagent dissolved in ethanol

(10 mg/ml), was added and mixed by shaking on a vortex mixer. The mixture was then irradiated at 365 nm for 30 minutes with a UVGL 25 device at its long wavelength setting. The UVGL device is sold by UVP Inc., 5100 Walnut Grove Avenue, P.O. Box 1501, San Gabriel, CA 91778, U.S.A.

(ii) The sample was also labeled with N-(4-azido-2-nitrophenyl)-N'-(N-d-biotinyl-3-aminopropyl)-N'-methyl-1,3-propanediamine (commercially available from BRESA, G.P.O. Box 498, Adelaide, South Australia 5001, Australia), following the procedure described by Forster et al (1985), *supra* for DNA.

(iii) When unlysed cells were used, the cell suspension in 0.2 ml 10 mM borate was incubated with the photoreagent for 1 hour prior to irradiation.

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Example 11: Hybridization of the Products of Examples 8 and 10

Prior to hybridization, the membrane with spots of denatured unlabeled probe DNA was treated for up to 2 hours with a "prehybridization" solution to block sites in the membrane itself that could bind the hybridization probe. This and the hybridization solution, which also contained denatured labeled sample DNA, was comprised of approximately 0.9 M Na⁺, 0.1% SDS, 0.1-5% bovine serum albumin or nonfat dry milk, and optionally formamide. With 50% formamide, the prehybridization and hybridization steps were done at approximately 42°C; without, the temperature was approximately 68°C. Prehybridized membranes can be stored for some time. DNA hybridization was allowed to occur for about 10 minutes or more, then unbound labeled DNA was washed from the membrane under conditions such as 0.018 M Na⁺ (0.1 × SSC), 0.1% SDS, 68°C, that dissociate poorly base paired hybrids. After posthybridization washes, the membrane was rinsed in a low salt solution without detergent in anticipation of hybridization detection procedures.

Example 12: Detection of a Nucleic Acid Hybrid with Immunogold

Affinity isolated goat antibiotin antibody (purchased from Zymed Laboratories, San Francisco, California, U.S.A.) was adsorbed onto colloidal gold (20 nm) following the method described by its supplier (Janssen instruction booklet, Janssen Life Sciences Products, Piscataway, New Jersey, U.S.A.) and reacted with hybridized biotinylated DNA after blocking as in a colorimetric method. The signals were silver enhanced using a Janssen (B2340 BEERSE, Belgium) silver enhancement kit and protocol.

Example 13: Detection of Urinary Tract Infection in a Urine Sample

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Urine samples were collected from a hospital where they were analyzed by microbiological methods and the results were kept secret until the hybridization diagnosis was conducted. Then they were compared ascertain the validity of the hybridization results.

1 ml of clinical sample (urine) suspected of UTI infection was centrifuged in a Brinkman micro centrifuge for 5 minutes. Then 0.1 ml of 1.2 N sodium hydroxide was added and the suspension was heated to 100°C to lyse the cells. The suspension was then diluted to 1 ml with 10 mM sodium borate buffer pH 8 and was neutralized with hydrochlorine acid to a pH of 7. To the solution, 50 µg "biotin-PEG-angelicin" (see Example 2) is added and the mixture was irradiated with a UVL 56 long wavelength UV lamp for 15 minutes. The irradiated sample (0.1 ml) was added to 3 ml 3XSSC of 5% nonfat dry milk 10% PEG with 0.2 M sodium pyrophosphate and hybridization was conducted with probes (whole genomic DNA) immobilized onto nitrocellulose paper at 68°C for 5 minutes to overnight. After hybridization detection was conducted according to Examples 3 or 12, the spots or the photographs were visually interpreted for the presence of specific bacteria in the test sample. A spot of human DNA was also present in the nitrocellulose paper for the detection of leucocytes. The presence of leucocytes was further verified with a common method using "LEUKOSTIX" (Miles Laboratories, Elkhart, Indiana, U.S.A.).

Typical results (Tables 1 and 2) indicate that the hybridization diagnosis produces similar results in a shorter time then the corresponding microbiological assays. The present invention not only provides information related to species identification, but also the leucocyte content in a clinical sample.

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TABLE 1

DIAGNOSIS OF CLINICAL URINE SAMPLES

* HOSPITAL DIAGNOSIS	APPLICANTS' HYBRIDIZATION RESULTS	DETECTION SYSTEM
NEG NEG NEG NEG NEG NEG NEG	NEG NEG NEG E.c.-M E.c.-VW E.c.-VW	GOLD GOLD GOLD CHEMI GOLD GOLD
S+, C- S+, C- S+, C- S+, C- S+, C- S+, C- S+, C- S+, C- S+, C- S+, C- S+, C-	NEG E.c.-S E.c.-S, Kl.-M NEG NEG NEG E.c.-VW NEG E.c.-VW NEG NEG	GOLD CHEMI CHEMI GOLD GOLD GOLD GOLD GOLD GOLD GOLD GOLD
100,000/mL E.c. 100,000/mL E.c. 100,000/mL E.c. 50,000/mL E.c. 50,000/mL E.c. E. coli E. coli E. coli E. coli/Klebsiella mix E. coli/Staph mix	E.c.-S E.c.-S E.c.-W E.c.-M NEG E.c.-S, Kl.-M E.c.-VS, Kl.-S E.c.-S, Kl.-S E.c.-S, Kl.-W E.c.-S, St.-M	GOLD CHEMI GOLD CHEMI GOLD CHEMI CHEMI CHEMI GOLD CHEMI

50

55

Table 1 cont'd

HOSPITAL DIAGNOSIS	APPLICANTS' HYBRIDIZATION RESULTS	DETECTION SYSTEM
Klebsiella spp.	E.c.-M, Kl.-W	CHEMI
100,000/mL K. pneumoniae	E.c.-W, Kl.-VW	GOLD
Enterobacter spp.	NEG**	GOLD
100,000 Candida	NEG**	GOLD
100,000/mL Proteus	Pr.-S, E.c.-W	GOLD
<hr/>		
10,000/mL Strep	NEG	CHEMI
Mixture of 3 unidentified Gm(+)	NEG	GOLD
<hr/>		
<p>* diagnosis conducted by streaking urine on an agar plate and treating the plate under conditions so that the infectious organism can grow.</p>		
<p>** Enterobacter/Candida probes not included in the hybridization assay, therefore, negative results are not surprising; given the high stringency conditions employed in the assay, cross-hybridization with species related to Enterobacter was not detected.</p>		
<p>Abbreviations: VS=very strong; S=strong; M=medium; W=weak; and VW=very weak hybridization signals; GOLD=detection method according to Example 12; CHEMI=chemiluminescent detection according to Example 3 (b)</p>		

Applicants' hybridization results represent the results of a subjective interpretation of the intensity of the hybridization signals obtained after detection. DNAs from the organisms listed in column two are the only ones for which any hybridization signal was obtained. The panel of DNAs used for hybridization included E. coli ("E.c."), Klebsiella pneumoniae ("Kl"), Proteus vulgaris ("Pr"), Pseudomonas aeruginosa, Staphylococcus epidermatis ("SE"), Streptococcus faecalis and Homo sapiens.

5

TABLE 2

COMPARISON OF AMES LEUKOSTIX ASSAY
WITH APPLICANT'S ASSAY

10	<u>"LEUKOSTIX"</u> <u>RESULT</u>	<u>APPLICANTS' HYBRIDIZATION</u> <u>RESULT</u>	<u>DETECTION</u> <u>SYSTEM</u>
	3+	VS	GOLD
15	3+	S	CHEMI
	3+	S	CHEMI
	3+	M	CHEMI
	3+	M	CHEMI
	3+	S	GOLD
20	3+	S	GOLD
	3+	VS	GOLD
	3+	VS	GOLD
	3+	VS	GOLD
	3+	VS	GOLD
25	-----		
	2+	S	CHEMI
	2+	S	CHEMI
30	2+	S	CHEMI
	2+	S	CHEMI
	2+	S	GOLD
	2+	S	GOLD
	2+	S	GOLD
35	2+	S	GOLD
	2+	S	GOLD
40	1+	S	GOLD
	1+	VS	GOLD
	1+	VW	GOLD
	TRACE/1+	M	CHEMI
	TRACE	VS	CHEMI
45	TRACE	W	GOLD
	TRACE	W	GOLD
	TRACE	VS	GOLD

	NEG	S	CHEMI
	NEG	S	CHEMI
	NEG	M	CHEMI
55	NEG	VW	GOLD

Table 2 cont'd

5	"LEUKOSTIX" RESULT	APPLICANTS' HYBRIDIZATION RESULT	DETECTION SYSTEM
	NEG	VW	GOLD
	NEG	NEG	GOLD
	NEG	NEG	GOLD
10	NEG	W	GOLD
	NEG	W	GOLD
	NEG	W	GOLD

15 The hybridization results summarized in column 2 of Table 2 represent subjective interpretations of the intensity of hybridization signal obtained when labeled urine samples described in Table 1 were hybridized with genomic human DNA.

The "LEUKOSTIX" assay is a colorimetric reagent strip assay. Color development on the reagent strip is compared to a chart provided with the assay reagent strips and ranges from negative (no color development) to 3+ (very strong color development).

Example 14: Lysis of Cells

25 A 1.0 mL aliquot of cell suspension was centrifuged and the cell pellet resuspended in 100 μ L of unbuffered NaOH solution. The sample was then exposed to high temperature for a short time and then diluted to the original volume using 10 mM borate buffer. The pH of the solution was then adjusted to neutral with HCl.

30 Table 4 shows the efficiency of lysis of two different Gram positive cocci, *Staphylococcus epidermidis* and *Streptococcus faecalis*, at varying NaOH concentrations at either 68°C or 100°C. In this Example, the absorbance of the 10 mL aliquots at 600 nm was recorded before centrifugation. After centrifugation, the cell pellets were resuspended in varying concentrations of NaOH (100 μ L) and duplicate samples of each exposed to 68°C for 10 minutes or 100°C for 5 minutes. Each sample was then diluted to 1.0 mL and the absorbance at 600 nm again recorded. Since the beginning and ending volumes are identical, the beginning and ending absorbance at 600 nm provides a direct measurement of lysis efficiency.

35 Whereas Gram negative organisms lysed efficiently in as low as 0.1 N NaOH, Table 4 shows clearly that efficient lysis is a function of both NaOH concentration and temperature, such that higher NaOH concentrations are required as the incubation temperature decreases. At 100°C (maximum temperature at 1 atmosphere) a concentration of at least 1.6 N NaOH was required for efficient lysis of *S. epidermidis* and *S. faecalis*.
40 If lower temperatures are desirable or necessary, then higher concentrations of NaOH will be required to maintain lysis efficiency.

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TABLE 3

EFFICIENCY OF LYSIS OF GRAM POSITIVE BACTERIA
AT VARIOUS CONCENTRATIONS OF NaOH AT 68°C and 100°C

Streptococcus faecalis

	[NaOH]	100°C/5 Minutes			68°C/10 Minutes		
		OD600 PRE	OD600 POST	%LYSIS	OD600 PRE	OD600 POST	%LYSIS
10	0 N	0.475	0.366	23	0.512	0.357	30
	0.1	.509	.261	50	.513	.238	54
	0.2	.512	.194	62	.514	.259	50
	0.4	.504	.175	65	.513	.150	71
15	0.8	.506	.113	78	.505	.147	71
	1.2	.498	.082	84	.498	.150	70
	1.6	.487	.061	88	.426	.099	77

Staphylococcus epidermidis

	[NaOH]	100°C/5 Minutes			68°C/10 Minutes		
		OD600 PRE	OD600 POST	%LYSIS	OD600 PRE	OD600 POST	%LYSIS
20	0 N	0.667	0.558	16	0.690	0.560	19
	0.1	.681	.396	42	.701	.441	37
25	0.2	.674	.296	60	.699	.414	41
	0.4	.699	.183	74	.730	.309	58
	0.8	.705	.091	87	.715	.187	74
	1.2	.680	.070	90	.719	.090	88
30	1.6	.693	.035	95	.660	.040	94

It will be appreciated that the instant specification and claims are set forth by way of illustration and not limitation, and that various modifications and changes may be made without departing from the spirit and scope of the present invention.

Claims

1. A method for detecting one or more microorganisms or polynucleotide sequences from eukaryotic sources in a nucleic acid-containing test sample comprising
 - a) preparing a test sample comprising labeling the nucleic acids in the test sample,
 - b) preparing one or more probes by immobilizing an oligonucleotide or a single-stranded nucleic acid of one or more known microorganisms or sequences from eucaryotic sources,
 - c) contacting, under hybridization conditions, the labeled single-stranded sample nucleic acid and the immobilized oligonucleotide or single-stranded nucleic acid to form hybridized labeled nucleic acids, and
 - d) assaying for the hybridized nucleic acids by detecting the label.
2. A method according to claim 1, further comprising denaturing the labeled nucleic acids to form labeled single stranded nucleic acids.
3. A method according to any of claims 1 and 2, wherein said eukaryotic sources are selected from the group consisting of algae, protozoa, fungi slime molds and mammalian genetic defects, such as alpha-thalassemia and sickle cell anemia.
4. A method according to any of claims 1 to 3, wherein the labeling is conducted in a whole living cell or a cell lysate.
5. A method according to claim 4, wherein the cell lysate is prepared by contacting a cell with alkali.
6. A method according to any of claims 1 to 5, wherein the label is selected from the group consisting of protein binding ligands, haptens, antigens, fluorescent compounds, dyes, radioactive isotopes and enzymes.
7. A method according to any of claims 1 to 6, wherein the immobilization is carried out by chemical reaction or physical adsorption.

8. A method according to any of claims 1 to 7, wherein the probe comprises the two or more known microorganisms or sequences from eukaryotic sources immobilized in the form of dots on a solid support strip.

9. A method according to any of claims 1 to 8, wherein said labeling is carried out by photochemically reacting a nucleic acid binding ligand with the nucleic acid in the test sample.

A kit for detecting one or more microorganisms or polynucleotide sequences from eukaryotic sources in a test sample comprising in one or more containers

a) a solid support containing single-stranded nucleic acids of one or more known microorganisms or polynucleotide sequences from eukaryotic sources immobilized thereon,

b) a reagent for labeling the nucleic acid of the test sample,

c) a reagent for denaturing nucleic acid in the test sample, and

d) hybridization reagents.

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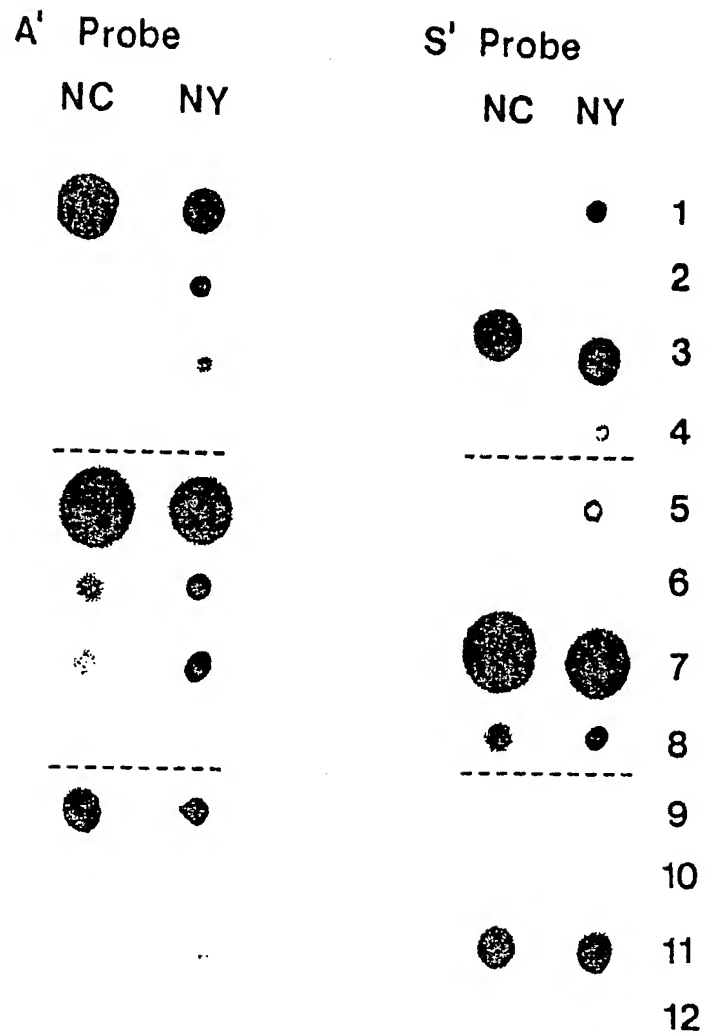
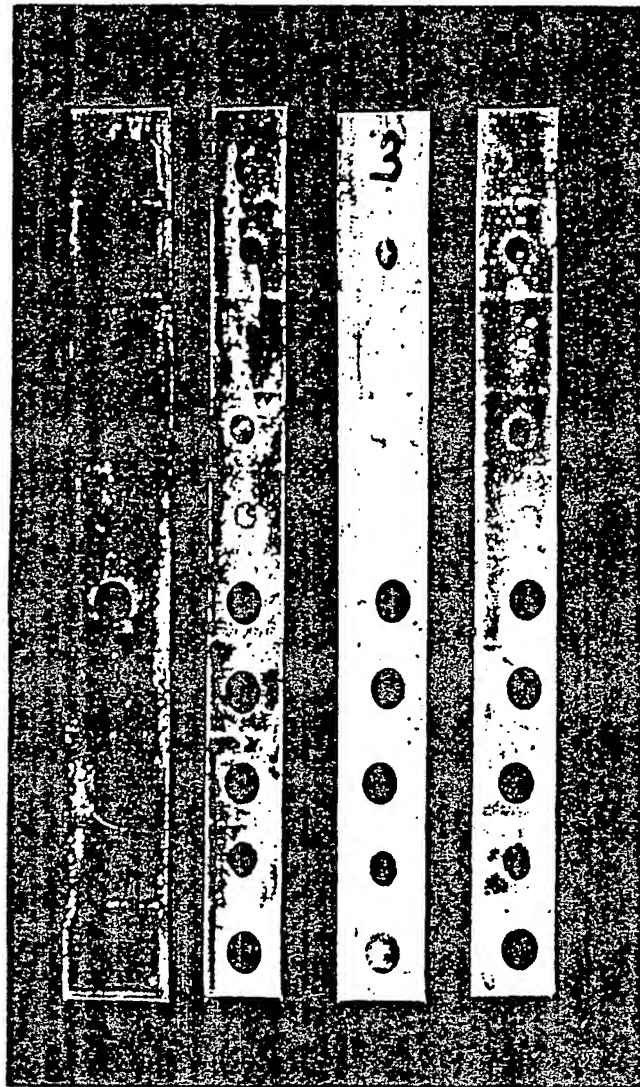


FIG. 1

A-K
A
S-K
S



45 50 57 60 deg.

FIG. 2

Brandt, Alan

From: Brandt, Alan
Sent: Thursday, August 24, 2006 2:48 PM
To: 'Wirtzberger, Paul Anthony'
Subject: RE: REMINDER - FW: REMINDER - FW: REMINDER - FW: CONFIDENTIAL: Draft US Util Pat App - Your ref: HP PD 200313056-1 ; Our ref: 28250.04109

Paul,

Thanks for your attention to this matter.

I have a few revisions to the application due to comments from the managing HP attorney (Mr. Gehman).

After addressing these comments, I will provide you and the other inventors with the revised draft application and Declaration and Assignment papers for execution.

Regards,
Al

-----Original Message-----

From: Wirtzberger, Paul Anthony [mailto:paul.wirtzberger@hp.com]
Sent: Thursday, August 24, 2006 1:58 PM
To: Brandt, Alan
Subject: RE: REMINDER - FW: REMINDER - FW: REMINDER - FW: CONFIDENTIAL: Draft US Util Pat App - Your ref: HP PD 200313056-1 ; Our ref: 28250.04109

I have reviewed the application and it looks good, I have no changes.
I also checked with the other two engineers and they have no changes.

paul

Hello all,

We acknowledge receipt of comments on the draft application from Mr. Gehman, managing HP attorney.

However, we have not yet received comments from the inventors.

Inventors - Please provide comments at your earliest convenience.

Please let us know if you have any questions.

Regards,
Al

ALAN BRANDT
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8/25/2006

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-----Original Message-----

From: Brandt, Alan

Sent: Tuesday, July 25, 2006 6:50 PM

To: 'Paul A. Wirtzberger (paul.wirtzberger@hp.com)'; 'Gary Williams (gary.williams@hp.com)'; 'Rico Brooks (rico.brooks@hp.com)'

Cc: 'Peggy Oyama (peggy.oyama@hp.com)'; 'Les Gehman (les.gehman@hp.com)'; Pejic, Ned

Subject: REMINDER - FW: REMINDER - FW: CONFIDENTIAL: Draft US Util Pat App - Your ref: HP PD 200313056-1 ; Our ref: 28250.04109

-----Original Message-----

From: Brandt, Alan

Sent: Wednesday, July 12, 2006 2:31 PM

To: Paul A. Wirtzberger (paul.wirtzberger@hp.com); Gary Williams (gary.williams@hp.com); Rico Brooks (rico.brooks@hp.com)

Cc: Peggy Oyama (peggy.oyama@hp.com); Les Gehman (les.gehman@hp.com)

Subject: REMINDER - FW: CONFIDENTIAL: Draft US Util Pat App - Your ref: HP PD 200313056-1 ; Our ref: 28250.04109

Inventors,

Please advise when you might be able to provide comments on the attached draft patent application.

Regards,
Al

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-----Original Message-----

From: Brandt, Alan

Sent: Wednesday, June 14, 2006 2:38 PM

To: 'Paul A. Wirtzberger (paul.wirtzberger@hp.com)'; 'Gary Williams (gary.williams@hp.com)'; 'Rico Brooks (rico.brooks@hp.com)'

Cc: Pejic, Ned; 'Peggy Oyama (peggy.oyama@hp.com)'; 'Les Gehman (les.gehman@hp.com)'

Subject: CONFIDENTIAL: Draft US Util Pat App - Your ref: HP PD 200313056-1 ; Our ref:

8/25/2006

28250.04109

Paul, Gary & Rico,

Attached is a draft of the subject U.S. utility patent application. The specification and claims are in the WORD document and the drawings are in the PowerPoint file. A table identifying the names associated with certain drawing reference numbers is also attached for your convenience to assist in going between the text and drawings. If applicable, please also distribute a copy of the application to any additional co-inventors for review. Additionally, please coordinate collective feedback on changes or comments from all co-inventors.

Only individuals that have contributed to the claims can be listed as inventors on the application. Please call me if there needs to be a change in inventorship (either deletions or additions). Otherwise, if applicable, please have each inventor confirm that they have contributed to some portion of at least one of the claims by identifying the claim(s) and corresponding inventor(s).

Please also carefully review the application to make sure that it describes the current best mode of the invention. As you may know, each inventor has an obligation to disclose to the US Patent & Trademark Office (USPTO) any information that may be material to the examination of the application including any prior art of which the inventor may be aware. Hence, please forward any such materials to me for filing with the USPTO.

The HP attorney has requested an inventor-approved draft application by the end of June. Therefore, we request your review of the draft application at your earliest convenience so that we can meet this deadline. Please let us know if you will have be able to review the applications within this timeframe so that we can provide the HP attorney with early notice of a delay in the inventor-approved draft.

If you have any questions, please do not hesitate to contact us. Thank you for your time and cooperation in this matter.

Regards,

AI

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Brandt, Alan

From: Brandt, Alan
Sent: Thursday, August 24, 2006 12:59 PM
To: 'jbertrand@invacare.com'
Cc: Pejic, Ned; Raulerson, Billy; Hinton, Jennifer; Zitelli, William
Subject: FW: MK6 CONTROLLER UTILITY PAT APP STATUS thru 8/24/06

John,

Updated status through mid-day 8/24/06.

I understand that you were to meet with the decision maker on foreign filing this morning. Currently, you have instructed us to file all 8 applications (5220, 5244, 5245, 5246, 5247, 5248, 5258, 5391) in Canada, two via PCT (5247 & 5258). Please let us know if these foreign filing instructions change.

Preparation of two draft utility applications continues (5244 & 5391). All other drafts (5220, 5245, 5246, 5247, 5248, 5258) are complete.

Calfee's internal review process is complete for three (5247, 5248, 5258) of four draft applications. The fourth application (5245) remains in the internal review process.

One draft application is with Invacare inventors for review (5220).

One application has been approved by Invacare for filing (5246). US Declaration/POA and Assignment papers have been executed for this application.

If anyone has any updates to the status, please let me know.

Regards,

Al

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8/25/2006



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁴ : C12Q 1/68	A1	(11) International Publication Number: WO 89/11548 (43) International Publication Date: 30 November 1989 (30.11.89)
(21) International Application Number: PCT/US89/02170 (22) International Filing Date: 18 May 1989 (18.05.89) (30) Priority data: 197,000 20 May 1988 (20.05.88) US 347,495 4 May 1989 (04.05.89) US (71) Applicant: CETUS CORPORATION [US/US]; 1400 Fifty-Third Street, Emeryville, CA 94608 (US). (72) Inventors: SAIKI, Randall, K. ; 320 39th Street, Richmond, CA 94805 (US). ERLICH, Henry, A. ; 3936 Rhoda Ave- nue, Oakland, CA 94602 (US). (74) Agent: HALLUIN, Albert, P.; Cetus Corporation, 1400 Fifty-Third Street, Emeryville, CA 94608 (US).		(81) Designated States: AT (European patent), AU, BE (Euro- pean patent), CH (European patent), DE (European pa- tent), DK, FI, FR (European patent), GB (European pa- tent), IT (European patent), JP, LU (European patent), NL (European patent), NO, SE (European patent). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
(54) Title: IMMOBILIZED SEQUENCE-SPECIFIC PROBES (57) Abstract <p>An improved nucleic acid hybridization assay reagent capable of binding a nucleic acid having a selected target sequence comprises a solid support matrix having oligonucleotide probes covalently attached thereto via a spacer arm. In a preferred embodiment, the solid support has reactive amino groups capable of binding UV irradiated nucleotides in an oligonucleotide probe, which probe comprises a hybridizing region complementary to a target sequence to be detected and a linking tail, the tail being composed of nucleotides, one or more of which is covalently bonded to the reactive groups of said support.</p>		

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IMMOBILIZED SEQUENCE-SPECIFIC PROBES

This invention relates to nucleic acid chemistry and to methods for detecting particular nucleic acid sequences. More specifically, the invention relates to a method for immobilizing DNA and RNA probes, stable assay reagents comprising the immobilized probes, and hybridization assays conducted with these immobilized probes. The invention has applications in the fields of medical diagnostics, medical microbiology, forensic science, environmental monitoring of microorganisms, food and drug quality assurance, and molecular biology.

Investigational microbiological techniques have been applied to diagnostic assays. For example, Wilson *et al.*, U.S. Patent No. 4,395,486 discloses a method for detecting sickle cell anemia by restriction fragment length polymorphism (RFLP). Wilson *et al.* identified a restriction enzyme capable of cleaving a normal globin gene but incapable of cleaving the mutated (sickle cell) gene. As sickle cell anemia arises from a point mutation, the method is effective but requires 10 to 20 ml of blood or amniotic fluid.

Various infectious diseases can be diagnosed by the presence in clinical samples of specific DNA sequences characteristic of the causative microorganism or infectious agent. Pathogenic agents include certain bacteria, such as Salmonella, Chlamydia, and Neisseria; viruses, such as the hepatitis, HTLV, and HIV viruses; and protozoans, such as Plasmodium, responsible for malaria. U.S. Patent No. 4,358,535 issued to Falkow *et al.* describes the use of specific DNA hybridization probes for the diagnosis of infectious diseases. The Falkow *et al.* method for detecting pathogens involves spotting a sample (e.g., blood, cells, saliva, etc.) on a filter (e.g., nitrocellulose), lysing the cells, and fixing the DNA through chemical denaturation and heating. Then, labeled DNA probes are added and allowed to hybridize with the fixed sample DNA, and hybridization indicates the presence of the pathogen DNA. A problem inherent in the Falkow *et al.* procedure is insensitivity; the procedure does not work well when very few pathogenic organisms are present in a clinical sample from an infected patient or when the DNA to be detected constitutes only a very small fraction of the total DNA in the sample. Falkow *et al.* do teach that the sample DNA may be amplified by culturing the cells or organisms in place on the filter.

Routine clinical use of DNA probes for the diagnosis of infectious diseases would be simplified considerably if non-radioactively labeled probes could be employed as

described in EP 63,879 to Ward. In the Ward procedure, horseradish peroxidase (HRP) labeled DNA probes are detected by a chromogenic reaction similar to ELISA. The Ward detection methods and reagents are convenient but relatively insensitive, again because the specific sequence that must be detected is usually present in extremely small quantities.

5 A significant improvement in DNA amplification, the polymerase chain reaction (PCR) technique, was disclosed by Mullis in U.S. Patent No. 4,683,202, and detection methods utilizing PCR are disclosed by Mullis *et al.* in U.S. Patent No. 4,683,195. In the PCR technique, short oligonucleotide primers are prepared which match opposite ends of a sequence to be amplified. The sequence between the primers need not be known. A sample of DNA or RNA is extracted and denatured, preferably by heat. Then,
10 oligonucleotide primers are added in molar excess, along with dNTPs and a polymerase, preferably Taq polymerase, which is stable to heat and commercially available from Perkin-Elmer/Cetus Instruments. DNA polymerase is "primer-directed," in that replication initiates at the two primer annealing sites. The DNA is replicated, and then again denatured.

This replication results in two "long products," which begin with the respective
15 primers, and the two original strands (per duplex DNA molecule). The products are called "long products," only because there is no defined point of termination of the synthesized strand. The reaction mixture is then returned to polymerizing conditions (e.g., by lowering the temperature, inactivating a denaturing agent, and, if necessary, adding more polymerase), and a second cycle initiated. The second cycle provides the two original
20 strands, the two long products from cycle one, two new long products (replicated from the original strands), and two "short products" replicated from the long products produced in cycle one. The products are called "short products," because these strands must terminate at the 5' end of the "long product" template -- the end defined by the primer that initiated synthesis of the long product. The short products contain the sequence of the target
25 sequence (sense or antisense) with a primer at one end and a sequence complementary to a primer at the other end. On each additional cycle, an additional two long products are produced, and a number of short products, equal to the number of long and short products remaining at the end of the previous cycle, are also produced. Thus, the number of short products can double with each cycle. This exponential amplification of a specific target
30 sequence allows the detection of extremely small quantities of DNA.

The PCR process has revolutionized and revitalized the nucleic acid based medical diagnostics industry. Because the present invention provides reagents that will often be

utilized in conjunction with PCR, some additional background information on PCR may be helpful. The PCR process can be used to amplify any nucleic acid, including single or double-stranded DNA or RNA (such as messenger RNA), nucleic acids produced from a previous amplification reaction, DNA-RNA hybrids, or a mixture of any of these nucleic acids. If the original or target nucleic acid containing the sequence variation to be amplified is single stranded, its complement is synthesized by adding one or more primers, nucleotides, and a polymerase; for RNA, this polymerase is reverse transcriptase.

The PCR process is useful not only for producing large amounts of one specific nucleic acid sequence, but also for amplifying simultaneously more than one different specific nucleic acid sequence located on the same or different nucleic acid molecules. When one desires to produce more than one specific nucleic acid sequence in PCR, the appropriate number of different oligonucleotide primers are utilized. For example, if two different specific nucleic acid sequences are to be produced, four primers can be utilized: two for each specific nucleic acid sequence to be amplified.

The specific nucleic acid sequence amplified by PCR can be only a fraction of a larger molecule or can be present initially as a discrete molecule, so that the specific sequence amplified constitutes the entire nucleic acid. In addition, the sequence amplified by PCR can be present initially in an impure form or can be a minor fraction of a complex mixture, such as a portion of nucleic acid sequence due to a particular microorganism that constitutes only a very minor fraction of a particular biological sample. The nucleic acid or acids to be amplified may be obtained from plasmids such as pBR322, from cloned DNA or RNA, or from natural DNA or RNA from sources such as bacteria, yeast, viruses, and higher organisms such as plants or animals. DNA or RNA may be extracted from blood or tissue material such as chorionic villi or amniotic cells by a variety of techniques, including the well known technique of proteolysis and phenol extraction, as is common for preparation of nucleic acid for restriction enzyme digestion. In addition, suitable nucleic acid preparation techniques are described in Maniatis *et al.*, Molecular Cloning: A Laboratory Manual (New York, Cold Spring Harbor Laboratory, 1982), pp. 280-281; U.S. Patent Nos. 4,683,195 and 4,683,202; EP 258,017; and Saiki *et al.*, 1985, Biotechnology 3:1008-1012.

Any specific nucleic acid sequence can be produced by the PCR process. It is only necessary that a sufficient number of bases at both ends of the sequence be known in sufficient detail so that two oligonucleotide primers can be prepared which will hybridize to

different strands of the desired sequence at relative positions along the sequence such that an extension product synthesized from one primer, when it is separated from its template (complement), can serve as a template for extension of the other primer. The greater the knowledge about the bases at both ends of the sequence, the greater can be the specificity of the primers for the target nucleic acid sequence, and thus the greater the probability that the process will specifically amplify the target.

The specific amplified nucleic acid sequence produced by PCR is produced from a nucleic acid containing that sequence and called a template or "target." If the target nucleic acid contains two strands, the strands are separated before they are used as templates, either in a separate step or simultaneously with the synthesis of the primer extension products. This strand separation can be accomplished by any suitable denaturing method, including physical, chemical, or enzymatic means. One physical method of separating the nucleic acid strands involves heating the nucleic acid until it is completely (>99%) denatured. Typical heat denaturation involves temperatures ranging from about 80 to 105°C for times ranging from about 1 second to 10 minutes. Strand separation may also be induced by a helicase enzyme, or an enzyme capable of exhibiting helicase activity, e.g., the enzyme RecA, which has helicase activity and in the presence of riboATP is known to denature DNA. The reaction conditions suitable for separating the strands of nucleic acids with helicases are described in Cold Spring Harbor Symposia on Quantitative Biology, Vol. XLIII "DNA: Replication and Recombination" (New York, Cold Spring Harbor Laboratory, 1978), B. Kuhn *et al.*, "DNA Helicases", pp. 63-67, and techniques for using RecA are reviewed in Radding, 1982, Ann. Rev. Genetics 16:405-437.

When the complementary strands of the nucleic acid or acids are separated, whether the nucleic acid was originally double or single stranded, the strands are ready to be used as a template for the synthesis of additional nucleic acid strands. The amplification reaction is generally conducted in a buffered aqueous solution, preferably at a pH of 7 to 9 (all pH values herein are at room temperature) most preferably about pH 8. Preferably, a molar excess (for cloned nucleic acid, usually about 1000:1 primer:template, and for genomic nucleic acid, usually about 10⁶⁻⁸:1 primer:template) of the two oligonucleotide primers is added to the buffer containing the separated template strands. The amount of complementary strand may not be known, however, in many applications, so that the amount of primer relative to the amount of complementary strand may not be determinable with certainty.

The deoxyribonucleoside triphosphates dATP, dCTP, dGTP, and dTTP are also added to the PCR mixture in adequate amounts, and the resulting solution is heated to about 90-100°C for about 1 to 10 minutes, preferably from 1 to 4 minutes. If the target nucleic acid forms secondary structure, the nucleotide 7-deaza-2'-deoxyguanosine-5'-triphosphate is also employed, as is known in the art, to avoid the potential problems such secondary structure can cause. After heating, the solution is allowed to cool to room temperature, preferred for primer hybridization. To the cooled mixture is added a polymerization agent, and the polymerization reaction is conducted under conditions known in the art. This synthesis reaction may occur at temperatures primarily defined by the polymerization agent. Thus, for example, if an *E. coli* DNA polymerase is used as a polymerizing agent, the maximum temperature for polymerization is generally no greater than about 40°C. Most conveniently, the reaction using *E. coli* polymerase occurs at room temperature. For most PCR applications, however, the thermostable enzyme Taq polymerase is employed at much higher temperatures, typically 50 to 70°C.

Nevertheless, the polymerization agent for PCR may be any compound or system, including enzymes, which will function to accomplish the synthesis of primer extension products from nucleotide triphosphates. Suitable enzymes for this purpose include, for example, *E. coli* DNA polymerase I, Klenow fragment of *E. coli* DNA polymerase I, T4 DNA polymerase, other available DNA polymerases, reverse transcriptase (used in the first cycle of PCR if the target is RNA), and other enzymes, including heat-stable enzymes such as Taq polymerase, which will facilitate combination of the nucleotides in the proper manner to form the primer extension products which are complementary to a target nucleic acid strand. Generally, synthesis will be initiated at the 3' end of each primer and proceed in the 5' direction along the template strand, until synthesis terminates. There may be agents, however, which initiate synthesis at the 5' end and proceed in the other direction, and there seems no reason such agents could not also be used as polymerization agents in PCR.

The newly synthesized strand in PCR is base paired to a complementary nucleic acid strand to form a double-stranded molecule, which in turn is used in the succeeding steps of the PCR process. In the next step, the strands of the double-stranded molecule are separated to provide single-stranded molecules on which new nucleic acid is synthesized. Additional polymerization agent, nucleotides, and primers may be added if necessary for the reaction to proceed. The PCR steps of strand separation and extension product

synthesis can be repeated as often as needed to produce the desired quantity of the specific nucleic acid sequence.

As noted above, PCR has revolutionized the nucleic acid based diagnostics industry. European Patent Office Publication 237,362, incorporated herein by reference, discloses assay methods employing PCR. In EP 237,362, PCR-amplified DNA is fixed to a filter and then treated with a prehybridization solution containing SDS, Ficoll, serum albumin, and various salts. A specific oligonucleotide probe (of e.g., 16 to 19 nucleotides) is then added and allowed to hybridize. Preferably, the probe is labeled to allow for detection of hybridized probes. EP 237,362 also describes a "reverse" dot blot, in which the probe, instead of the amplified DNA, is fixed to the membrane.

The recent advent of PCR technology has enabled the detection of specific DNA sequences initially present in only minute (<1 ng) quantities. For example, Higuchi *et al.*, 1988, *Nature* 332:543-546, describe the characterization of genetic variation between individuals based on samples containing only a single hair. DNA was isolated from the hair by digestion and extraction and then treated under PCR conditions to obtain amplification. Specific nucleotide variations were then detected by either fragment length polymorphism (PCR-FLP), hybridization to sequence-specific oligonucleotide (SSO) probes (a technique also described in Saiki *et al.*, 1986, *Nature* 324:163-166) or by direct sequencing via the dideoxy method (using amplified DNA rather than cloned DNA). Because PCR results in the replication of a DNA sequence positioned between two primers, insertions and deletions between the primer sequences result in product sequences of different lengths, which can be detected by sizing the product in PCR-FLP. In SSO hybridization, the amplified DNA can be fixed to a nylon filter by UV irradiation in a series of "dot blots" and, in one variation of the technique, then allowed to hybridize with an oligonucleotide probe labeled with HRP under stringent conditions. After excess probe is removed by washing, 3, 3', 5, 5'-tetramethylbenzidine (TMB) and H₂O₂ are added: HRP catalyzes H₂O₂ oxidation of TMB to a blue precipitate, the presence of which indicates hybridized probe. U.S. Patent No. 4,789,630, incorporated herein by reference, describes protocols and TMB compounds useful for purposes of the present invention. One may alternatively use one of the other leuco dyes (such as a red leuco dye developed by DuPont and licensed to Kodak) to indicate the presence of HRP. However, any chromogen that develops precipitable color or fluorescence as a consequence of peroxidatic activity can be used to detect HRP-labeled reagents. In fact, any enzyme can be used to label, so long as

there exists a colorless substrate which forms a colored or fluorescent product as a result of enzyme activity and the product can be captured on a solid support. Separate dot blot hybridizations are performed for each allele tested.

Church *et al.*, 1984, *Proc. Natl. Acad. Sci. USA* 81:1991-1995, discloses a method for genomic sequencing which comprises cross-linking restriction enzyme-digested genomic DNA fragments to nylon membranes using UV irradiation, and probing the bound fragments with comparatively long (100-200 bp) DNA probes. Church *et al.* also discloses that NTPs dried onto nylon membranes and UV irradiated at 0.16 KJ/m² for two minutes are bound more stably (i.e., TTP = 130x, dGTP = 30x, dCTP = 20x, and dATP = 10x) than non-UV irradiated nucleotides. Primary amino groups are highly reactive with 254 nm light-activated thymine (see Saito *et al.*, 1981, *Tetrahedron Lett.* 22:3265-68), and this reactivity is believed to be the mechanism by which nucleotides become covalently bound to a membrane.

The detection of genetic variations using SSO probes is typically performed by first denaturing and immobilizing the sample DNA on a nylon or nitrocellulose membrane. The membrane is then treated with short (15-20 base) oligonucleotides under stringent hybridization conditions, allowing annealing only in cases of exact complementarity. A large number of hybridizations must be performed when a sample is examined for the presence of many different sequences. For example, a test for the most common genetic mutations that lead to beta-thalassemia in Mediterranean populations would involve 12 probes and require 12 separate hybridizations, accomplished either by probing one filter 12 times or by conducting simultaneous hybridizations on 12 replicate filters (or some combination thereof). A DNA-based HLA typing test can require 20 to 50 probes and hybridizations, a prohibitive effort if one uses the prior art methods that require either splitting the sample into as many portions as there are probes or blotting the sample followed by probing with a single probe and then removing the probe, a process that must be repeated for each probe tested.

In traditional nucleic acid detection by oligomer hybridization, the DNA in the test sample, including the hybridization target, is noncovalently chemisorbed onto a solid support such as nitrocellulose or nylon and then hybridized to a labeled target-specific probe which, except in the SSO methodology just described, usually contains hundreds to thousands of nucleotides and is made biosynthetically. This method suffers from multiple deficiencies. The noncovalent target capture generally is weak enough that considerable

target may be washed from the solid support during detection (see, for example, Gingeras *et al.*, 1987, Nucleic Acids Research 15:5373-5390 and Gamper *et al.*, 1986, Nucleic Acids Research 14:9943-9954). Target chemisorption reduces the reactivity of the target sequence toward hybridization with probe. The capture and hybridization processes normally take many hours to reach completion. The need to chemisorb the target immediately before detection prevents the manufacture of a storage-stable capture reagent with built-in target specificity that can be applied rapidly to test samples. The sequence non-specificity of capture complicates the examination of a single test sample with more than one probe: either a lot of test sample must be available to load different solid supports to incubate with the various probes or a lot of time must be consumed in serial probing of a singly immobilized test sample.

Ranki *et al.*, 1983, Gene 21:77-85, improve on the traditional technology by creating a sequence-specific capture reagent, capturing the target sequence from the test sample by nucleic acid hybridization and increasing specificity by detecting captured target with a second, labeled, sequence-specific nucleic acid probe. However, their technology still suffers from multiple deficits. The sequence-specific capture probe is immobilized by chemisorption, so that the assay still is vulnerable to signal attenuation by desorption of both capture probe and probe-target complex during the incubations and washes. Chemisorption reduces probe reactivity, requiring long incubation times to maximize capture efficiency. Two nucleic acid probes must be manufactured instead of one. The capture and detection probes of Ranki *et al.* are so large that they must be prepared by biosynthetic instead of much cheaper chemical synthetic routes. A small capture probe would not be immobilized efficiently by chemisorption.

Gingeras *et al.*, 1987, *supra*, improve further on DNA probe technology by covalently attaching relatively short, chemically synthesized, oligonucleotide hybridization probes to a solid support, dramatically reducing hybridization time. However, direct coupling of the target-specific sequence to the support risks reduced reactivity caused by steric occlusion by the support. Furthermore, the method demonstrated no way of detecting captured DNA apart from the incorporation of radioactive label into the target, a procedure which is relatively hazardous and inconvenient. Finally, the beaded solid support of Gingeras *et al.* is hard to adapt to assays in which multiple targets are probed, because the test sample must be exposed to separate containers of beads carrying the different probes, taking care not to mix beads with different specifications.

Gamper *et al.*, *supra*, describe a different strategy to accelerate oligomer hybridization: oligo hybridization is performed in solution rather than on a solid support, the hybrid species being simultaneously photochemically trapped, because the target-specific oligomer has been chemically modified with a moiety which crosslinks double-stranded DNA when irradiated. However, apart from the expense of creating the photo-adduct labeling reagent, this method suffers the inconvenience and delay associated with
5 ultrafiltration to remove the considerable excess of unreacted probe, followed by gel electrophoresis to purify the hybridization product to the point that it can be identified. This procedure would be particularly inconvenient to adapt to simultaneous probing of multiple targets, because of the need to engineer targets to be electrophoretically resolvable.

10 The present invention provides a particularly advantageous assay method which permits the simultaneous nonisotopic detection of two or more specific nucleic acid sequences or control conditions in a single test sample, using a single solid support divided into discrete regions to which different oligonucleotide probes have been covalently attached via spacer arms. The method comprises:

- 15 (a) attaching the probes to defined regions of the solid support through spacer arms, attached at one end to the probe and at the other end to the support;
- (b) reacting the test sample with the probe-bearing solid support under conditions promoting hybridization of the probes to any single-
20 stranded complementary nucleic acid sequences in the test sample;
- (c) washing away any nucleic acid not hybridized to probe; and
- (d) detecting the probe-captured nucleic acid, preferably nonisotopically.

25 Because of the permanence of covalent attachment, the preparation of immobilized probes can be separated in time from their use, permitting manufacture of a storage-stable detection reagent, the hybridization capture support, which can be used rapidly to detect target nucleic acid sequences in test samples on demand. Covalent probe attachment and the use of a spacer arm between support and probe greatly accelerate and improve the efficiency of hybridization. The use of a dimensionally stable solid support with discrete regions for
30 different probes greatly improves the economics, simplifies the physical format, and increases the reliability of hybridization and detection, because all target sequences in a simple test sample and all control conditions can be probed simultaneously in a single short

incubation and because all probe-target hybrids are exposed to identical incubation, wash, and detection conditions. Nonisotopic detection, whether via colored or fluorescent labels directly attached to the target nucleic acid or via colored, fluorescent, or enzyme labels indirectly attached to the target nucleic acid through a specific binding reaction, is much safer and more convenient than detection of radioactive atoms attached to the target nucleic acid, especially when developing storage-stable detection reagents and assay kits.

The invention also relates to a novel, stable, assay reagent comprising oligonucleotide probes covalently attached to discrete regions of a solid support via spacer arms, which probes have sequences designed to hybridize to different analyte nucleic acids in the test sample or to indicate different (positive or negative) control conditions which test the validity of the assay conditions. This assay reagent will have significant commercial impact, being ideally suited to large-scale, automated, manufacturing processes and having a long shelf life. The reagent will prove especially useful in situations where the number of target sequences exceeds the number of samples tested. In general, the greater the number of target sequences and therefore immobilized probes, the greater the improvement of the invention over what has gone before. With PCR-amplified DNA samples, a simple test can easily be assayed for over fifty specific sequences on a single solid support. The nonisotopic detection aspect of the invention is especially well suited to target sequences generated by PCR, a process which permits covalent attachment to all target molecules of colored or fluorescent dyes and of binding moieties like biotin, of colored or fluorescent and of binding moieties like biotin, digoxin, and specific nucleic acid sequences. The methods and reagents of the invention are also suited to detection of isotopically labeled nucleic acid, although this mode is not preferred.

An important aspect of the invention relates to a specific chemistry for attaching oligonucleotide probes to a solid support in a way which is especially suitable to large-scale manufacture and which permits maximization of probe retention and hybridization efficiency. This chemistry comprises covalent attachment of a polynucleotide (preferably poly-dT) tail to the probe and fixation by the ultraviolet irradiation of the photoreactive tailed probe to a solid support bearing primary or secondary amines (e.g., a nylon membrane). However, the invention provides numerous alternative, non-photochemical ways to attach probe to support, wherein electrophilic reagents are used to couple the probe to the spacer and the spacer to the solid support in either reaction order, and wherein the spacer can be any of a large variety of organic polymers or long-chain compounds.

Another aspect of the invention relates to a DNA sequence detection kit, which kit comprises the stable assay reagent, essentially a solid support having oligonucleotide probes covalently bound thereto via a spacer arm. The kit can also include PCR reagents, including PCR primers selected for amplification of DNA sequences capable of hybridizing with the oligonucleotide probes.

5 To aid in understanding and describing the invention, the following terms are defined below:

"Allele-specific oligonucleotide" (ASO) refers to a probe that can be used to distinguish a given allelic variant from all other allelic variants of a particular allele by hybridization under sequence-specific hybridization conditions.

10 "DNA polymorphism" refers to the condition in which two or more different variations of a nucleotide sequence exist in the same interbreeding population.

"Genetic disease" refers to specific deletions and/or mutations in the genomic DNA of an organism that are associated with a disease state and include sickle cell anemia, cystic fibrosis, alpha-thalassemia, beta-thalassemia, and the like.

15 "Label" refers to any atom or molecule which can be used to provide a detectable (preferably quantifiable) signal and which can be attached to a nucleic acid or protein. Labels may provide signals detectable by fluorescence, radioactivity, colorimetry, X-ray diffraction or absorption, magnetism, enzymatic activity, and the like. Suitable labels include fluorophores, chromophores, radioactive atoms (particularly ^{32}P and ^{125}I), electron-
20 dense reagents, enzymes, and ligands having specific binding partners. Enzymes are typically detected by their activity. For example, HRP can be detected by its ability to convert diaminobenzidine (more preferably, however, TMB is used) to a blue pigment, quantifiable with a spectrophotometer. It should be understood that the above description is not meant to categorize the various labels into distinct classes, as the same label may
25 serve in several different modes. For example, ^{125}I may serve as a radioactive label or as an electron-dense reagent. HRP may serve as enzyme or as antigen for an antibody, such as a monoclonal antibody (MAb). Further, one may combine various labels for desired effect. For example, MAbs and avidin can be labeled and used in the practice of this invention. One might label a probe with biotin, and detect its presence with avidin labeled
30 with ^{125}I or with an anti-biotin MAb labeled with HRP. Alternatively, one may employ a labeled MAb to dsDNA (or hybridized RNA) and thus directly detect the presence of hybridization without labeling the nucleic acids. Other permutations and possibilities will

be readily apparent to those of ordinary skill in the art and are considered within the scope of the instant invention.

"Oligonucleotide" refers to primers, probes, oligomer fragments, oligomer controls, and unlabeled blocking oligomers and is a molecule comprised of at least two or more deoxyribonucleotides or ribonucleotides. An oligonucleotide can also contain
5 nucleotide analogues, such as phosphorothioates and alkyl phosphonates, and derivatized (i.e., labeled) nucleotides. The exact size of an oligonucleotide will depend on many factors, which in turn depend on the ultimate function or use of the oligonucleotide.

"Primer" refers to an oligonucleotide, whether occurring naturally or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed
10 under conditions in which synthesis of a primer extension product complementary to a nucleic acid strand can occur. The primer is preferably an oligodeoxyribonucleotide and is single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. The primer must be sufficiently long to prime the
15 synthesis of extension products in the presence of the polymerase, but the exact length of a primer will depend on many factors. For example, for diagnostics applications, the oligonucleotide primer typically contains 15 to 25 nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with template. Suitable primers for amplification are prepared by means known to those of
20 ordinary skill in the art, for example by cloning and restriction of appropriate sequences, direct chemical synthesis, and purchase from a commercial supplier. Chemical methods for primer synthesis include: the phosphotriester method described in Narang *et al.*, 1979, *Meth. Enzymol.* 68:90 and U.S. Patent No. 4,356,270; the phosphodiester method disclosed in Brown *et al.*, 1979, *Meth. Enzymol.* 68:109; the diethylphosphoramidite
25 method disclosed in Beaucage *et al.*, 1981, *Tetrahedron. Lett.* 22:1859-1862; and the solid support method disclosed in U.S. Patent No. 4,458,066. The primers may also be labeled, if desired.

"Restriction fragment length polymorphism" (RFLP) refers to a DNA polymorphism at a restriction enzyme recognition site. The restriction enzyme specific for
30 the polymorphic site can be used to digest sample DNA, and when the digested DNA is fractionated by electrophoresis and, if necessary, treated for visualization, different samples

produce different restriction endonuclease patterns, depending on the particular polymorphic sequence present in the sample.

"Sequence-specific hybridization" refers to strict hybridization conditions in which exact complementarity between probe and sample target sequence is required for hybridization to occur. Such conditions are readily discernible by those of ordinary skill in the art and depend upon the length and base composition of the probe. In general, one may vary the temperature, pH, ionic strength, and concentration of chaotropic agent(s) in the hybridization solution to obtain conditions under which substantially no probes will hybridize in the absence of an "exact match." For hybridization of probes to bound DNA, the empirical formula for estimating optimum temperature under standard conditions (0.9 M NaCl) is: $T(^{\circ}\text{C}) = 4(N_G + N_C) + 2(N_A + N_T) - 5^{\circ}\text{C}$, where N_G , N_C , N_T , and N_A are the numbers of G, C, A, and T bases in the probe (J. Meinkoth *et al.*, 1984, *Analyt. Biochem.* 138:267-284). Those of skill in the art recognize, however, that this calculation only gives an approximate value for optimum temperature, which should then be empirically tested to obtain the true optimum temperature. The probe utilized in a sequence-specific hybridization is called a "sequence-specific oligonucleotide" (SSO), which can also be an allele-specific oligonucleotide (ASO). Those of skill in the art recognize that for a single mismatch between probe and target to be destabilizing, the hybridizing region of the probe must be relatively short, generally no longer than about 23 bases, and usually about 17 to 23 bases in length.

"Specific binding partner" refers to a protein capable of binding a ligand molecule with high specificity, as for example in the case of an antigen and an antibody or MAb specific therefor. Other specific binding partners include biotin and avidin, streptavidin, or an anti-biotin antibody; IgG and protein A; and the numerous receptor-ligand couples known in the art.

To aid in understanding the invention, several Figures accompany the description of the invention. These Figures are briefly described below.

Figure 1 depicts a plot of probe binding as a function of UV exposure.

Figure 2 depicts a plot of hybridization efficiency as a function of UV exposure.

Figure 3 depicts a series of dot blots demonstrating the presence of either normal beta-globin or sickle cell beta-globin, obtained by sandwich assay.

Figure 4 depicts a series of dot blots demonstrating the presence of either normal beta-globin or sickle cell beta-globin, obtained by direct assay.

Figure 5 depicts a series of dot blots demonstrating HLA DQalpha genotyping.

Figure 6 depicts a series of dot blots demonstrating beta-thalassemia typing.

The present invention provides a method for detecting the presence of a specific nucleotide sequence in a sample by contacting the sample with immobilized oligonucleotide probes under conditions that allow for hybridization of complementary nucleic acid sequences. In one embodiment of the method, the test sample is contacted with a solid support upon which are immobilized probes specific for one or more target sequences (the "analyte"), probes for a positive control sequence that should be present in all test samples, and optionally probes for a negative control sequence that should not be present in any test sample -- each different probe is immobilized at a distinct region on the solid support. If an analytical signal is detected in the negative control region or if no analytical signal is detected in the positive control region, then the validity of the response in the analyte region is suspect, and the sample should be retested. In another preferred embodiment, a number of different analyte-specific probes are covalently attached to distinct regions of a solid support so that one can test for allelic variants of a given genetic locus in a single hybridization reaction. In still another preferred embodiment, the various analyte-specific probes are complementary to nucleotide sequences present in various microorganisms.

The immobilized probes are also an important aspect of the invention. The probes comprise two parts: a hybridizing region composed of a nucleotide sequence of about 10 to 50 nucleotides (nt) and a spacer arm, at least as long as the hybridizing region, which is covalently attached to the solid support and which acts as a "spacer", allowing the hybridizing region of the probe to move away from the solid support, thereby improving the hybridization efficiency of the probe. In a preferred embodiment, the spacer arm is a sequence of nucleotides, called the "tail", that serves to anchor the probes to the solid support via covalent bonds between nucleotides within the tail of the probe and reactive groups within the solid support matrix.

As described more fully below, the immobilized probes of the invention avoid the problems inherent in prior art detection methods with immobilized probes. These problems include a lack of sensitivity, for many prior art methods for immobilizing probes actually result in the hybridizing region of the probe becoming attached to the solid support and thus less free to hybridize to complementary sequences in the sample. In addition, the prior art methods for synthesizing and attaching the spacer to the probe and to the solid support are complicated, time-consuming, expensive, and often involve the use of toxic reagents. In

marked contrast, a preferred method of the invention for synthesizing and immobilizing probes is quickly completed with readily available, relatively nontoxic, reagents, and with practically no chemical manipulations. The polynucleotide tails of the invention are composed of nucleotides that are attached to the hybridizing region with an enzyme or with the aid of commercially available nucleic acid synthesizers. The tails of the invention are attached to the solid support by a similarly problem-free method: exposure to ultraviolet (UV) light.

Those of skill in the art recognize that nucleic acid hybridization serves as the basis for a number of important techniques in the medical diagnostics and forensics industries. In addition, nucleic acid hybridization serves as an important tool in the laboratories where scientific advances in many diverse fields occur. The present invention represents an important step in making nucleic acid based diagnostics even more powerful and useful. As noted above, PCR has played an important role in these same industries and laboratories, and the present invention will often be practiced on samples in which the nucleic acid has been amplified by PCR. Various useful embodiments of the present invention are described below, but the full scope of the invention can only be realized when understood and utilized by the various and diverse practitioners of nucleic acid based diagnostics.

One very important use of the present invention relates to the detection and characterization of specific nucleic acid sequences associated with infectious diseases, genetic disorders, and cellular disorders, including cancer. In these embodiments of the invention, amplification of the target sequence is again useful, especially when the amount of nucleic acid available for analysis is very small, as, for example, in the prenatal diagnosis of sickle cell anemia using DNA obtained from fetal cells. Amplification is particularly useful if such an analysis is to be done on a small sample using non-radioactive detection techniques which may be inherently insensitive, or where radioactive techniques are being employed but where rapid detection is desirable.

The immobilized probes provided by the present invention not only are useful for detecting infectious diseases and pathological abnormalities but also are useful in detecting DNA polymorphisms not necessarily associated with a pathological state. The term forensic is most often used in a context pertaining to legal argument or debate. Individual identification on the basis of DNA type is playing an ever more important role in the law. For example, DNA typing can be used in the identification of biological fathers and so

serves as an important tool for paternity testing. DNA typing can also be used to match biological evidence left at the scene of a crime with biological samples obtained from an individual suspected of committing the crime. In a similar fashion, DNA typing can be used to identify biological remains, whether those remains are a result of a crime or some non-criminal activity. The practice of forensic medicine now routinely involves the use of DNA probes, in protocols that can be made more efficient by employing the present invention.

To achieve the important and diverse benefits of the present invention, one must first synthesize the probes to be immobilized on a solid support. The probe sequence can be synthesized in the same manner as any oligonucleotide, and a variety of suitable synthetic methods were noted above in the discussion of PCR primers. The hybridizing region of the probes of the invention is typically about 10 to 50 nt in length, and more often 17 to 23 nt in length, but the exact length of the hybridizing region will of course depend on the purpose for which the probe is used. Often, for reasons apparent to those of skill in the art, the hybridizing region of the probe will be designed to possess exact complementarity with the target sequence to be detected, but once again, the degree of complementarity between probe and target is somewhat tangential to the present invention. The probes of the invention are, however, preferably "tailed" with an oligonucleotide sequence that plays a critical role in obtaining the benefits provided by the present invention.

This tail of the probes of the invention consists of ribonucleotides or deoxyribonucleotides (e.g., dT, dC, dG, and dA). The nucleotides of the tail can be attached to the hybridizing region of the probe with terminal deoxynucleotidyl transferase (TdT) by standard methods. In addition, the entire tailed probe can be synthesized by chemical methods, most conveniently by using a commercially available nucleic acid synthesizer. One can also synthesize the tails and hybridizing regions separately and then combine the two components. For instance, a preparation of tails can be prepared (and even attached to a solid support, such as a bead) and then attached to a preparation of hybridizing regions.

When using a DNA synthesizer to make the tailed probes of the invention, one should take steps to avoid making a significant percentage of molecules that, due to a premature chain termination event, do not contain a hybridizing region. One such step involves synthesizing the hybridizing region of the probe first, creating a tailed probe with

the hybridizing region at the 3' end of the molecule. Because the likelihood of a premature chain termination event increases with the length of the molecule, this step increases the likelihood that if a chain termination event occurs, the occurrence merely results in a shorter tail. However, because most premature chain termination events are a result of a failure to "de-block" during synthesis, and because the exact number of residues in the tail of a probe of the invention is not critical, one may also merely omit the blocking and de-blocking steps during automated synthesis of the tail region of the probe. If these steps are omitted (during tail synthesis only), the tail can be placed on either the 5' or 3' end of the probe with equal efficiency and satisfactory results.

As noted above, the preferred spacer arms of the probes of the invention are comprised of nucleotide tails. Because the tails serve to attach the probe to the solid support, the relative efficiency with which a given oligonucleotide will react with a solid support is important in choosing the sequence to serve as the tail in the probes of the invention. Most often, the tail will be a homopolymer, and Figure 1, below, depicts the relative efficiencies with which synthetic oligonucleotides with varying length homopolymer tails were covalently bound to a nylon filter as a function of UV exposure. As shown in the figure, oligonucleotides with longer poly-dT tails were more readily fixed to the membrane, and all poly-dT tailed oligonucleotides attained their maximum values by 240 mJ/cm² of irradiation at 254 nm. In contrast, the poly-dC (400 nt in length) tail required more irradiation to crosslink to the membrane and was not comparable to the equivalent poly-dT tail even after 600 mJ/cm² exposure. Untailed oligonucleotides were retained by the filter in a manner roughly parallel to that of the poly-dC-tailed probes.

Thus, the probes of the invention preferably comprise a poly-dT tail of greater than 10 thymidine (T) residues. Usually, the tail will comprise at least 100 T residues, and most preferably, the tail will comprise at least 400 dT nucleotides. As the poly-dT tail functions primarily to bind the probe to the solid support, the exact number of dT nucleotides is not critical, as noted above. Although those of skill in the art will readily recognize the fact, it should be noted that the composition of the tail need not be homogeneous, i.e., a mixture of nucleotides may be used. Preferably, however, the tail will include a significant number of thymine bases, as T reacts most readily with the solid support by the preferred methods for making the immobilized probes of the invention, which methods are discussed more fully below. If one desires to utilize the probes in sequence-specific hybridizations, one must be aware of the problem caused by creation of a random sequence in the

heterogeneous tail that closely resembles the hybridizing region of a probe. If a heterogeneous tail is employed, it is still desirable to maintain a distribution of 150 pyrimidine residues per tail, if the probes are to be fixed to a solid support by UV irradiation.

5 The tail should always be larger than the hybridizing region, and the greater the disparity in size between the hybridizing region and the tail (so long as the tail is larger), the more likely it is that the tail, rather than the hybridizing region, will react with the support, a preferred condition. Larger tails thus increase the likelihood that only the tail will participate in reacting with and thereby binding to the solid support. Because the tail also functions as a "spacer," enabling the complementary sequence to diffuse away from the
10 solid support where it may hybridize more easily, free of steric interactions, larger tails are doubly preferred. Excessively extended tails, however, are uneconomical, and, if carried to an extreme, excessive tailing could have adverse effects.

A preferred method of synthesizing a probe of the invention is as follows. The probe is synthesized on a DNA synthesizer (the Model 8700, marketed by Biosearch, is
15 suitable for this purpose) with beta-cyanoethyl N, N-diisopropyl phosphoramidite nucleosides (available from American Bionetics) using the protocols provided by the manufacturer. If desired, however, only the hybridizing region of the probe is synthesized on the instrument, and then 200 pmol of the probe are tailed in 100 μ l of reaction buffer at pH = 7.6 and containing 100 mM cacodylate, 25 mM Tris-base, 1 mM CoCl_2 , and 0.2 mM
20 dithiothreitol with 5 to 160 nmol deoxyribonucleotide triphosphate (dTTP) and 60 units (50 pmol) of terminal deoxyribonucleotidyl transferase (available from Ratliff Biochemicals) for 60 minutes at 37 degrees C (see Roydhouhury *et al.*, 1980, *Meth. Enz.* 65:43-62, for buffer preparation). Reactions are conveniently stopped by the addition of 100 μ l of 10 mM EDTA. The lengths of tails can be controlled by limiting the amount of dTTP (or other
25 nucleotide) present in the reaction mixture. For example, a nominal tail length of 400 dT residues is obtained by using 80 nmol of dTTP in the protocol described above.

Once the tailed probe of the invention is synthesized, the probe is then attached to a solid support. Suitable solid supports for purposes of the present invention will contain (or
30 can be treated to contain) free reactive primary or secondary amino groups capable of binding a UV-activated pyrimidine, especially thymine. Secondary amino groups may be preferred for purposes of the present invention. There are many ways to assure that a solid support (not necessarily nylon) has free, particularly secondary, amino groups.

Amine-bearing solid supports suitable for purposes of the present invention include polyethylenimine (chemisorbed to any solid, such as cellulose or silica with or without glutaraldehyde crosslinking) and silica or alumina or glass silanized with amine-bearing reagents such as PCR Inc.'s Prosil™ 220, 221, 3128, and 3202 reagents. Manville sells controlled porosity glass papers (Biomat™) appropriate for aminoalkyl silanization. One
5 may alkylate immobilized primary amines (e.g. with a methyl halide or with formaldehyde plus cyanoborohydride, (as described by Jentoff *et al.*, 1979, *J. Biol. Chem.* 254:4359-4365). As noted above, one may use a solid support to which polyethylenimine has been chemisorbed. Polyvinyl chloride sheets containing PEI-loaded silica are commercially available (manufactured by Amerace and sold by ICN as Protrans™ and by Polysciences
10 as Poly/Sep™), and PEI loading of cellulose is well known.

The solid support, also called a substrate, can be provided in a variety of forms, including membranes, rods, tubes, wells, dipsticks, beads, ELISA-format plates, and the like. A preferred support material is nylon, which contains reactive primary amino groups and will react with pyrimidines irradiated with UV light. Preferred solid supports include
15 charge modified nylons, such as the Genetrans-45™ membrane marketed by Plasco and the ZetaProbe™ membrane marketed by Bio-Rad.

Having chosen a suitable solid support, one makes the preferred immobilized probes of the invention by reacting a tailed oligonucleotide probe with the solid support under conditions that favor covalent attachment of the tail to the solid support. In a
20 preferred embodiment, the solid support is a membrane, and probe binding results from exposure of the probe on the membrane to UV irradiation, which activates the nucleotides in the tail, and the activated nucleotides react with free amino groups within the membrane. Careful dessication of tailed probes of the invention spotted onto a suitable solid support can also be used to facilitate covalent attachment of the probes to the substrate. One can
25 assay for the presence or absence in a solid support matrix of reactive groups capable of reacting with oligonucleotides by the procedure described in Example 1.

As is apparent from the foregoing, a preferred method for preparing the immobilized probes of the invention comprises fixing an oligonucleotide probe with a poly-
30 dT tail to a nylon membrane by UV irradiation. Although poly dT tails react very efficiently to solid supports by the methods of the present invention, efficiency of reaction of oligonucleotides with a membrane does not necessarily correlate with hybridization efficiency. One may therefore wish to determine the hybridization efficiency of a given

oligonucleotide probe after immobilization on a solid support. When the hybridization of various tailed probes is measured as a function of UV dosage, as shown in Figure 2, one observes that the optimum exposure changes with length of a poly-dT tail. Optimal exposures are about 20 mJ/cm² for 800 nt poly-dT tails and about 40 mJ/cm² for 400 nt poly-dT tails.

5 At 60 mJ/cm² exposure, one observes that oligonucleotides with longer tails hybridize more efficiently than can be accounted for by the additional amounts of probe reacted with and bound to the filter. This increased efficiency is believed to be due to a spacing effect: increasing the distance between the membrane and the hybridizing region of the immobilized probe may increase hybridization efficiency of the probe. Thus, too much
10 UV exposure during immobilization can not only damage the nucleotides in the probe but also can reduce the average spacer length and decrease hybridization efficiency. It is important to note that because dC tails react less efficiently (as compared to dT tails) with a membrane, hybridization efficiency of a poly-dC tailed probe reaches a plateau where loss due to UV damage and tail shortening is compensated for by the fixing of new molecules
15 to the membrane (see Figures 1 and 2). This characteristic of poly-dC tails may make such tails preferred when UV exposure cannot be carefully controlled.

No matter what the base content of the tail of the probe, one may automate the attachment of probe to support in accordance with the method of the present invention. One semi-automated means of attachment preferred for positive charge nylon membranes is
20 as follows. A commercially available "dot-blot" apparatus can be readily modified to fit into a Perkin-Elmer/Cetus Pro/Pette® automated pipetting station; the membrane is then placed on top of the dot-blot apparatus and vacuum applied. The membrane dimples under the vacuum so that a small volume (5 to 20 µl) of probe applied forms a consistent dot with edges defined by the diameter of the dimple. No disassembly of the apparatus is required
25 to place and replace the membrane -- the vacuum may be kept constant while membranes are applied, spotted with probe, and removed.

Once the probes are spotted onto the membrane, the spotted membrane is treated to immobilize the probes. A preferred method for covalently attaching the probes to a nylon membrane is as follows. Tailed oligonucleotides in TE buffer (10 mM Tris-HCl, pH =
30 8.0, and 0.1 mM EDTA) are applied to a Genetrans-45™ (Plasco) membrane with a BioDot™ (Bio-Rad) spotting manifold. The damp membranes, also called "filters," are then placed on paper pads soaked with TE buffer; the pads and filters are then placed in a

UV light box (the Stratalinker 1800™ light box marketed by Stratagene is suitable for this purpose) and irradiated at 254 nm under controlled exposure levels. UV dosage can be controlled by time of exposure to a particular UV light source or, more preferably, by measuring the radiant UV energy with a metering unit. Exposure time typically ranges from about 0.1 to 10 minutes, most often about 2 to 3 minutes. The support is preferably damp during irradiation, but if the support is dried first, a shorter UV irradiation exposure can be used. The irradiated filters are washed in a large volume of a solution composed of 5X SSPE (1X SSPE is 180 mM NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA, pH = 7.2) and 0.5% sodium dodecyl sulfate (SDS) for about one-half hour at 55 degrees C to remove unreacted oligonucleotides. Filters can then be rinsed in water, air dried, and stored at room temperature until needed.

UV irradiation of nucleotides is known to cause pyrimidine photochemical dimerization, which, for purposes of the present invention, is not preferred. A number of steps can be taken to reduce dimerization during UV irradiation, including: applying the oligonucleotide probe to the membrane at a high pH, above 9 and preferably above 10; applying the probe to the membrane at a very low ionic strength, between 0 and 0.01; using the lowest probe concentration that gives the desired signal intensity; and irradiating with light excluding wavelengths longer than 250 nm, preferably with no light with a wavelength longer than 240 nm. However, the extent to which these steps could impair probe immobilization has not been tested. In general, spotting and attachment of the probe to the membrane should be done at a temperature and in a solvent that minimizes base-pairing and base-stacking in the probes.

After constructing the novel immobilized probes of the invention, one is ready to employ those probes in the useful nucleic acid sequence detection methods of the invention. In a preferred embodiment of this method, a sample suspected of containing a target nucleic acid sequence is treated under conditions suitable for amplifying the target sequence by PCR. Note that the process of "asymmetric" PCR, described by Gyllensten and Erlich, 1988, Proc. Natl. Acad. Sci. USA 85:7652-7656, for generation of single stranded DNA can also be used to amplify the sample nucleic acid. The PCR primers are biotinylated, for subsequent detection of hybridized primer-containing sequences. The amplification reaction mixture is denatured, unless asymmetric PCR was used to amplify, and then applied to a membrane of the present invention under conditions suitable for hybridization to occur (most often, sequence-specific hybridization). Hybridized probe is detected by

binding streptavidin-horseradish peroxidase (SA-HRP, available from a wide variety of chemical vendors) to the biotinylated DNA, followed by a simple colorimetric reaction in which a substrate such as TMB is employed. One can then determine whether a certain sequence is present in the sample merely by looking for the appearance of colored dots on the membrane.

5 In an especially preferred embodiment of the above method, a filter with immobilized oligonucleotides is placed in hybridization solution containing 5X SSPE, 0.5% SDS, and 100 ng/ml SA-HRP (as marketed under the SeeQuence™ by Cetus and Eastman Kodak). PCR-amplified sample DNA is denatured by heat or by addition of NaOH and EDTA and added immediately to the hybridization solution, which contains
10 enough SSPE to neutralize any NaOH present. The sample is then incubated at a suitable temperature for hybridization to occur (typically, as exemplified below, at 55 degrees C for 30 minutes). During this incubation, hybridization of product to immobilized oligonucleotide occurs as well as binding of SA-HRP to biotinylated product. The filters are briefly rinsed in 2X SSPE and 0.1% SDS at room temperature, then washed in the
15 same solution at 55-degrees C for 10 minutes, then quickly rinsed twice in 2X PBS (1X PBS is 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, and 8 mM Na₂HPO₄, pH = 7.4) at room temperature. Color development is performed by incubating the filters in red leuco dye or TMB at room temperature for 5 to 10 minutes. Photographs are taken of the filters after color development for permanent records.

20 Although the detection method described above is preferred, those of skill in the art recognize that the immobilized probes of the invention can be utilized in a variety of detection formats. One such format involves labeling the immobilized probe itself, instead of the sample nucleic acid. If the probe is labeled at or near the end of the hybridizing sequence (far from the site of attachment of the probe to the solid support), one can treat the
25 potentially hybridized sample DNA with an appropriate restriction enzyme, i.e., one that cleaves only duplex nucleic acids at a sequence present in the hybridizing region of the probe, so that restriction releases the label from the probe (and the membrane) for detection of hybridization. Suitable labels include peroxidase enzymes, acid phosphatase, radioactive atoms or molecules (e.g., ³²P, ¹²⁵I, etc.), fluorophores, dyes, biotin, ligands
30 for which specific monoclonal antibodies are available, and the like. If the primer or one or more of the dNTPs utilized in a PCR amplification has been labeled (for instance, the biotinylated dUTP derivatives described by Lo *et al.*, 1988, *Nuc. Acids Res.* 16:8719),

instead of the immobilized probe, then, as noted above, hybridization can be detected by assay for presence of label bound to the membrane.

The immobilized probes of the invention can also be used in detection formats in which neither probe nor primer is labeled. In such a format, hybridization can be detected using a labeled "second probe." The second probe is complementary to a sequence occurring within the target DNA, but not overlapping the bound probe sequence; after hybridization of the second probe, the immobilized probe, second probe, and target sequence form a nucleic acid "sandwich," the presence of which is indicated by the presence of the label of the second probe on the membrane. One could also employ monoclonal antibodies (or other DNA binding proteins) capable of binding specifically to duplex nucleic acids (e.g., dsDNA) in a detection format that uses no labeled nucleic acids. Those of skill in the art will recognize that one important advantage of the immobilized probes of the invention is the ability, at least with most detection formats, to recycle the support-bound probe by denaturing the hybridized complex, eluting the sample DNA, and treating the support (for example, by washing, bleaching, etc.) to remove any remaining traces of extraneous DNA, label, developer solution, and immobilized dye (see U.S. Patent No. 4,789,630 and PCT application No. 88/0287, incorporated herein by reference).

Those of skill in the art will recognize the many and diverse uses for the immobilized probes of the present invention. One exciting application of these immobilized probes is in conjunction with the technique of simultaneous amplification of several DNA sequences ("multiplex" PCR). Such simultaneous amplification can be used to type at many different loci with a single membrane. For instance, one can type for the polymorphic HindIII site in the Ggamma gene (see Jeffreys, 1979, Cell 18:1-10), the polymorphic AvaII site in the low density lipoprotein receptor gene (see Hobbs *et al.*, 1987, Nuc. Acids Res. 15:379), and for polymorphisms in the HLA DQalpha gene simultaneously by amplifying all three loci in a single PCR and applying the amplified material to a suitable set of immobilized probes of the present invention. Other genetic targets whose analysis would be simplified by this technique include the detection of somatic mutations in the ras genes, where six loci and 66 possible alleles occur (see Verlaan-de Vries *et al.*, 1986, Gene 50:313-320); the typing of DNA polymorphisms at the HLA DP locus; the detection of beta-thalassemia in Middle Eastern populations, where in addition to the endogenous mutations, Mediterranean and Asian Indian mutations are

present at significant frequencies; the detection of infectious pathogens; and the detection of microorganisms in environmental surveys.

In many of these applications, it will be desirable to obtain a membrane on which are immobilized a diverse set of oligonucleotides specific for different sequences that nevertheless can hybridize under the same sequence-specific hybridization conditions. If necessary, this situation can be achieved by adjusting the length, position, and strand-specificity of the probes, or by varying the amount of probe applied to the membrane, or by adding a salt, such as tetramethylammonium chloride, to the hybridization buffer to minimize differences among immobilized oligonucleotides caused by varying base compositions (see Wood *et al.*, 1985, *Proc. Natl. Acad. Sci. USA* 82:1585-1588).

The examples below illustrate various useful embodiments of the invention and enable the skilled artisan to appreciate the invention more fully and so should not be construed as limiting the scope of the invention in any way.

Example 1

Probe Retention and Hybridization Efficiency

The stable binding of poly-dT-tailed probe sequences to nylon as a function of tail length and UV exposure was examined as described below. A 19-base oligonucleotide (RS18: 5'-CTCCTGAGGAGAAGTCTGC) was labeled at its 5' end with gamma ^{32}P -ATP and T4 polynucleotide kinase (see Saiki *et al.*, 1986, *Nature* 324:163-166). Portions of the kinased probe were then tailed with dTTP and terminal transferase (TdT), as described by Roydhouhury *et al.* RS18 was present at a concentration of 2 μM , TdT (Ratloff Biochemicals, Los Alamos, NM) at 600 U/ml, and either dCTP or dTTP at either 0 μM , 50 μM , 100 μM , 200 μM , 400 μM , or 800 μM to prepare constructs with either dC or dT tails of approximately 0, 25, 50, 100, 200, 400 or 800 dT bases or 400 dC bases per molecule. Reaction mixtures were incubated for 60 minutes at 37°C and were terminated by addition of an equal volume of 10 mM EDTA.

Four pmol of each sample diluted in 100 μl of TE buffer were spotted onto nine duplicate filters (Genetrans-45 nylon, Plasco, Woburn, Mass.), UV irradiated for various times, washed to remove unbound oligonucleotides, and then each spot was measured by scintillation counting to determine the amount of probe crosslinked to the nylon membrane. The values plotted in Figure 1 are relative to an unirradiated, unwashed control filter (100% retention). UV irradiation was accomplished by placing the filters in a Stratalinker 1800™

UV light box and irradiating the filters at 254 nm. Dosage was controlled using the internal metering unit of the device. The filters were then washed in 5X SSPE, 0.5% SDS for 30 minutes at 55°C to remove DNA not stably bound. The results plotted in Figure 1 show that even the non-tailed probe was retained by the membrane, but that retention of the untail-
 5 exhibited >90% retention after suitable exposure.

However, high retention does not necessarily correlate with high hybridization efficiency. Thus, hybridization efficiency was measured as follows. Probes were prepared with poly-dT tails as above, but with unlabeled RS18. The probes were spotted onto filters and UV irradiated, and excess probe was washed from the membrane by
 10 incubating the membrane in 5X SSPE, 0.5% SDS, for 30 minutes at 55°C. The membranes were then hybridized with 5 pmol of complementary ³²P-kinase labeled 40-mer (RS24: 5'-CCCACAGGGCAGTAACGGCAGACTTCTCCTCAGGAGTCAG, specific activity of 1.5 uCi/pmol) in a solution (10 ml) containing 5X SSPE and 0.5% SDS, at 55°C for 20 minutes, which are sequence-specific hybridization conditions. The membrane was
 15 then washed first with 2X SSPE, 0.1% SDS (3 x 100 ml) for 2 minutes at about 25°C, then in 2X SSPE, 0.1% SDS at 55°C for 5 minutes. The individual spots were excised, counted, and the counts plotted against UV exposure, as shown in Figure 2. The values plotted are fmol RS24 hybridized to the membrane. The results show that none of the non-tailed probe was able to hybridize under the conditions used, even though as much as 50% of the applied RS18 should be bound to the membrane. All of the tailed probes were able
 20 to hybridize, with hybridization efficiency increasing with increasing tail length. Optimal UV exposures were from about 60 to 120 mJ/cm².

Example 2

Sandwich Assay for Sickle-Cell Anemia

Two allele-specific probes were prepared, one for the normal beta-globin allele, called RS18, and one for the sickle-cell allele, RS21 (5'CTCCTGTGGAGAAGTCTGC); each probe had a 400 nt poly-dT tail. If desired, a probe for the hemoglobin C allele can be prepared with the sequence: 5'CTCCTAAGGAGAAGTCTGC. Eight replicate filters
 30 were prepared and spotted with 4, 2, 1, and 0.5 pmol of each tailed probe using the method set forth in Example 1, and then UV irradiated by placing the filters, DNA-side down, directly onto a TM-36 Transilluminator UV light box (U.V. Products, San Gabriel, CA)

for 5 minutes. Four 1 µg samples of genomic DNA (from cell lines Molt4 (beta^Abeta^A), SC-1 (beta^Sbeta^S), M + S (beta^Abeta^S), and GM2064 (beta^Abeta^A), a beta-globin deletion mutant) were subjected to 30 PCR amplification cycles with the primer pair PC03 (5'ACACAACCTGTGTTCACTAGC) and KM38 (5'TGGTCTCCTTAAACCTGTCTTG).

5 PCR was carried out in substantial accordance with the procedure described by Saiki *et al.*, 1988, *Science* 239:1350-1354. The DNA samples were amplified in 100 µl of reaction buffer containing 50 mM KCl, 10 mM Tris-HCl (pH = 8.4), 1.5 mM MgCl₂, 100 µg/ml gelatin, 200 µM each of dATP, dCTP, dGTP, and dTTP, 0.2 µM of each primer, and 2.5 units of Taq DNA polymerase (Perkin-Elmer/Cetus Instruments). The cycling
10 reaction was performed on a programmable heat block, the DNA Thermal Cycler, available from PECL, set to heat at 95 degrees C for 15 seconds (denature), cool at 55 degrees C for 15 seconds (anneal), and incubate at 72 degrees C for 30 seconds (extend) using the Step-Cycle program. After 30 cycles, the samples were incubated an additional 5 minutes at 72 degrees C.

15 Each amplification product (18 µl) was denatured by heating at 95°C for 5 minutes in 1 ml of TE, and then quenched on ice. A solution (4 ml) of 6.25X SSPE, 6.25X Denhardt's, and 0.625% SDS was mixed with 1 ml of each denatured PCR product, hybridized to one of the filters for 15 minutes at 55°C, washed with 2X SSPE, 0.1% SDS (3 X 100 ml) for 2 minutes at about 25°C, and then washed with 2X SSPE, 0.1% SDS (1 x 100 ml) for 5 minutes at 55°C.

20 The membranes were then equilibrated in 2X SSPE, 0.1% (v/v) Triton X-100 (100 ml) for 3 minutes at about 25°C to remove SDS. All of the filters were then hybridized in the same buffer with a horseradish peroxidase (HRP) labeled 15-mer, RS111 (5'-GCAGGTTGGTATCAA), specific for the PC03/KM38 amplification product, prepared by the method disclosed in PCT publications WO 89/02931 and 89/02932, incorporated herein
25 by reference.

30 These methods essentially involve derivatizing the nucleic acid probe using a linear linking molecule comprising a hydrophilic polymer chain (e.g., polyoxyethylene) having a phosphoramidite moiety at one end and a protected sulfhydryl moiety at the other end. The phosphoramidite moiety couples to the nucleic acid probe by reactions well known in the art (e.g., Beaucage *et al.*, 1981, *Tetrahedron Lett.* 22:1859-1862), while the deprotected sulfhydryl group can form disulfide or other covalent bonds with a protein, e.g., HRP. The HRP is conjugated to the linking molecule through an N-maleimido-6-aminocaproyl

group. The label is prepared by esterifying N-maleimido-6-aminocaproic acid with sodium 4-hydroxy-3-nitrobenzene sulfonate in the presence of one equivalent of dicyclohexylcarbodiimide in dimethylformamide. After purification, the product is added to phosphate buffer containing HRP at a weight ratio of 8:1 HRP to ester. The oligonucleotide probe is synthesized in a DNA synthesizer, and the linking molecule having the structure $(C_6H_5)_3CS-(CH_2CH_2O)_4-P(CH_2CH_2CN)[N(i-Pr)_2]$ is attached using phosphoramidite synthesis conditions. The trityl group is removed, and the HRP derivative and probe derivative are mixed together and allowed to react to form the labeled probe. A biotin-labeled probe may be prepared by similar methods.

The membrane was incubated with 4 pmol RS111 in a solution (8 ml) composed of 5X SSPE, 5 x Denhardt's, and 0.5% Triton X-100 for 10 minutes at 40°C. Following incubation, the membrane was washed with 2X SSPE, 0.1% Triton X-100 (3 x 100 ml) for 2 minutes at about 25°C.

The reaction was followed by color development (Sheldon *et al.*, 1986, *Proc. Natl. Acad. Sci. USA* 83:9085-9089) with TMB/H₂O₂, as shown in Figure 3. The membranes were soaked in 100 ml of color development buffer B (CDB-B: 237 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8.0 mM Na₂HPO₄, pH 7.4, 5% (v/v) Triton X-100, 1 M urea, and 1% dextran sulfate), followed by washes with 2 x 100 ml of CDB-C (100 mM sodium citrate, pH 5.0) for 2 minutes at room temperature. Color was developed by replacing the CDB-C solution with 100 ml of CDB-D (100 mM sodium citrate, pH 5.0, 0.1 mg/ml 3,3',5,5'-tetramethylbenzidine), adding 50 µl of 3% H₂O₂, and allowing the color to develop for 30 minutes. The beta-globin genotypes of the amplified DNA samples were readily apparent from the filters, and good signal intensity was obtained even from the 0.5 pmol spot.

Example 3

Direct Assay for Sickle-Cell Anemia

A second set of DNAs (as described in Example 2 above) was amplified with PC03 and BW19. BW19 has the sequence 5'CAACTTCATCCACGTTTCACC, and is covalently bound to a molecule of biotin at the 5' end. Twelve µl of each of these amplification products were denatured as described in Example 2, added to 4 ml of hybridization buffer (6.25X SSPE, 6.25X Denhardt's, and 0.625% SDS), and incubated with the membrane-bound probe (the remaining four filters from Example 2 above) at 55°C for 15 minutes.

The membranes were then washed with 2X SSPE, 0.1% SDS (3 x 100 ml) for 3 minutes at room temperature, followed by a wash with 2X SSPE, 0.1% SDS (1 x 100 ml) for 5 minutes at 55°C.

5 The membranes were pooled together and equilibrated in 100 ml of CDB-A for 5 minutes at about 25°C (CDB-A: 237 mM NaCl, 2.7 mM KCl, 1.5 mM KH₂PO₄, 8.0 mM Na₂HPO₄, pH 7.4, and 5% Triton X-100). The membranes were then placed in a heat-sealable bag with 10 ml of CDB-A and See-Quence™ SA-HRP conjugate (Cetus Corporation, Emeryville, CA) at 0.3 µg/ml and gently shaken for 10 minutes at about 25°C. Excess conjugate was removed by washing with CDB-A (3 x 100 ml for 3 minutes at 25°C), CDB-B (1 x 100 ml for 5 minutes at 25°C), and CDB-C (2 x 100 ml for 3
10 minutes at 25°C). The membranes were then equilibrated in CDB-D (100 ml) for 5 minutes at room temperature, followed by addition of 50 µl of 3% H₂O₂. The color was allowed to develop for 30 minutes with gentle shaking, followed by washing under deionized water for 10 minutes. The four filters after color-development are shown in Figure 4. The intensity and specificity of signals detected by this method compare favorably to those
15 obtained by sandwich hybridization. The faint signal in GM2064 was due to beta-globin contamination in that DNA sample.

Example 4

HLA DQalpha Genotyping

20 The DQalpha test is derived from a PCR-based oligonucleotide typing system that partitions the polymorphic variants at the DQalpha locus into four DNA major types denoted DQA1, DQA2, DQA3, and DQA4, three DQA4 subtypes, DQA4.1, DQA4.2, and DQA4.3, and three DQA1 subtypes, DQA1.1, DQA1.2, and DQA1.3 (see Higuchi *et al.*, 1988, *Nature* 332:543-546 and Saiki *et al.*, 1986, *Nature* 324:163-166).

25 Four oligonucleotides specific for the major types, four oligonucleotides that characterized the subtypes, and one control oligonucleotide that hybridizes to all allelic DQalpha sequences were given 400 nt poly-dT tails and spotted onto 12 duplicate nylon filters. About 2 to 10 pmol of each probe were placed in each spot.

30 With regard to amount of probe spotted, however, one may wish to employ lower amounts of RH54 and GH64, i.e., 0.035 pmol RH54 per spot is preferred. These probes are positive control probes for amplification and will hybridize to any DQalpha alleles under the conditions described. By reducing the amount of positive control probe on the

membrane, one can make the positive control probe the least sensitive probe on the membrane. Then, if insufficient amplified DQalpha DNA is applied to the membrane, one can recognize the problem, for the positive control probe will not react or will react only very weakly. Otherwise, when insufficient sample DNA is applied to the membrane, one runs the risk of misreading a heterozygous type as a homozygous type, because some probes hybridize less efficiently than others.

After spotting, the membranes were irradiated at 40 mJ/cm². The sequences of the hybridizing regions of the resulting immobilized probes are shown below.

<u>DQA Type</u>	<u>Designation</u>	<u>Sequence</u>
A1	GH75	5'-CTCAGGCCACCGCCAGGCA or
	RH83	5'-GAGTTCAGCAAATTTGGAG
A2	RH71	5'-TTCCACAGACTTAGATTTG or
	RH82	5'-TTCCACAGACTTAGATTTGAC
A3	GH67	5'-TTCCGCAGATTTAGAAGAT
A4	GH66	5'-TGTTTGCCTGTTCTCAGAC
A1.1	GH88	5'-CGTAGAACTCCTCATCTCC
A1.2, 1.3, 4	GH89	5'-GATGAGCAGTTCTACGTGG
A1.3	GH77	5'-CTGGAGAAGAAGGAGAC
not A1.3	GH76	5'-GTCTCCTTCCTCTCCAG
all	RH54	5'-CTACGTGGACCTGGAGAG- GAAGGAGACTGCCTG or
	GH64	5'-TGGACCTGGAGAGGAAGGAGACTG
A4.2, A4.3, not A4.1	HE01	5'-CATCGCTGTGACAAAACAT

Although most of the probes are uniquely specific for one DQA type, two of the DQA1 subtyping probes cross hybridize to several DNA types. GH89 hybridizes to a sequence common to the DQA1.2, 1.3, and 4 types, and the probe GH76 detects all DQA types except DQA1.3. The GH76 probe is needed to distinguish DQA1.2/1.3 heterozygotes from DQA1.3/1.3 homozygotes. Further, the length and strand specificity of the probes were adjusted so that their relative hybridization efficiencies and stringency requirements for allelic discrimination were approximately the same. These eight probes produce a unique hybridization pattern for each of the 21 possible DQA diploid combinations.

The sequence variation that defines the DQalpha DNA types is localized within a relatively small hypervariable region of the second exon that can be encompassed within a single 242 bp PCR amplification product (see Horn *et al.*, 1988, Proc. Natl. Acad. Sci. USA 85:6012-6016). These primers are shown below.

Primer RS134 is 5'-GTGCTGCAGGTGTAAACTTGTACCAG

5

Primer RS135 is 5'-CACGGATCCGGTAGCAGCGGTAGAGTTG

Biotinylated PCR primers were used to amplify this 242 bp DQalpha sequence from several genomic DNA samples: six homozygous cell lines and six heterozygous individuals.

The biotinylated primers were synthesized as follows. Primary amino groups were introduced at the 5' termini of the primers by a variation of the protocols set forth in Coull *et al.*, 1986, Tetrahedron Lett. 27:3991-3994 and Connolly, 1987, Nuc. Acids Res. 15:3131-3139. Briefly, tetraethylene glycol was converted to the mono-phthalimido derivative by reaction with phthalimide in the presence of triphenylphosphine and diisopropyl azodicarboxylate (see Mitsunobu, 1981, Synthesis, pp. 1-28). The mono-phthalimide was converted to the corresponding beta-cyanoethyl diisopropylamino phosphoramidite as described in Sinha *et al.*, 1984, Nuc. Acids Res. 12:4539. The resulting phthalimido amidite was added to the 5' ends of the oligonucleotides during the final cycle of automated DNA synthesis using standard coupling conditions. During normal deprotection of the DNA (concentrated aqueous ammonia for five hours at 55 degrees C), the phthalimido group was converted to a primary amine which was subsequently acylated with an appropriate biotin active ester. LC-NHS-biotin (Pierce) was selected for its water solubility and lack of steric hindrance. The biotinylation was performed on crude, deprotected oligonucleotide and the mixture purified by a combination of gel filtration and reversed-phase HPLC (see Levenson *et al.*, 1989, in PCR Protocols and Applications - A Laboratory Manual, eds. Innis *et al.*, Academic Press, NY).

After hybridization of the amplified DNA to the membranes and color development, the DQalpha genotypes of these samples is readily apparent, as is shown in Figure 5. In Figure 5, the specificity of each immobilized probe is noted at the top of the filters and the DQA genotype of each sample is noted at the right of the corresponding filter.

The immobilized probes of the invention have so facilitated the method of DNA typing at the HLA DQalpha locus that kits for typing will be important commercially. These kits can come in a variety of forms, but a preferred embodiment of the kit is

described in detail, below. This description is followed by a description of simplified typing protocols for use with the kit.

A preferred kit will contain one or more vials of pre-aliquoted, "sterilized" (see below) DQalpha PCR amplification mixes, typically in concentrated (2X is preferred) form and pre-aliquoted in 50 μ l aliquots. Each 50 μ l aliquot will contain: 5 μ mol KCl, 1 μ mol Tris-HCl (pH = 8.3), 250 nmol $MgCl_2$, 15 pmol of biotinylated RS134, 15 pmol of biotinylated RS134, 18.75 nmol each of dGTP, dATP, dTTP, dCTP, and from 2.5 units up to 50 units of recombinant Taq polymerase (PECI). The dNTPs will be prepared from stock solutions at pH = 7. The sterilization protocol also introduces very low levels of inactivated DNase and NaCl, as noted below.

The "sterilized" reagents referred to above relate to the need to avoid contamination of reagents with non-sample-derived nucleic acid sequences. Because PCR is such a powerful amplification method, contaminating molecules can lead to error. To avoid this contamination problem, the present invention provides a novel sterilization procedure. This procedure employs a DNase, preferably bovine pancreas DNase, to remove low levels of DNA contamination from batches of PCR reaction mix. Because DNA primers are sensitive to this enzyme, the primers are omitted from the batch until the DNase has been inactivated by thermal denaturation. However, if RNA primers are to be employed in the PCR mixture, the primers can be present during sterilization. In addition, derivatized nucleotides can be used to make an oligonucleotide resistant to DNase; for instance, thio-substituted nucleotides, such as phosphorothioates can be used to prepare oligonucleotides resistant to DNase (see Sitzler and Eckstein, 1988, *Nuc. Acids Res.* 16:11,691). Those of skill in the art recognize that an equivalent sterilization procedure utilizes a restriction enzyme that cleaves a sequence present in the amplification target; if any contaminating target is present, the restriction enzyme will cleave the contaminant, rendering it unavailable for amplification.

In the preferred sterilization procedure, however, 2.5 ml of 10X Taq buffer (100 mM Tris-HCl, pH = 8.3; 500 mM KCl; and 25 mM $MgCl_2$) are autoclaved and added to 0.19 ml of a solution that is 25 mM in each dNTP, 0.13 ml of Taq DNA polymerase at a concentration of 5 U/ μ l, and 8.75 ml of glass distilled water. The mixing of these reagents can be conveniently carried out in a 50 ml polypropylene tube. Once the mixture is prepared, 650 U of DNase I (Cooper Biomedicals; 2500 U/ml in 150 mM NaCl, stored frozen) are added and the resulting solution incubated at 37 degrees C for 15 minutes. The

DNAse is inactivated by incubating the mixture at 93 degrees C for 10 minutes. Then, 0.38 ml of each primer at 10 μ M is added to the sterilized reagent, which is then aliquoted, preferably with a "dedicated" pipettor and in the protected confines of a laminar flow hood.

5 The kit can optionally contain the PCR reagents above, but must contain the immobilized probes of the invention, which can be prepared as described above with a blotting and automated pipetting device. The solid support can be conveniently marked by silk-screening. The kit can also contain SA-HRP at a concentration of 20 μ g/ml HRP, which correlates to 250 nmol/ml SA. The SA-HRP is supplied in a buffer composed of 10 mM ACES, 2 M NaCl, at a pH = 6.5. The kit can also contain a concentrated (5X to 20X) solution of chromogen, such as leuco dye (as is marketed by Kodak in the Sure-Cell™
10 diagnostic kits) or TMB.

The kit will also be more successful if simple, easy-to-follow instructions are included. Typical instructions for a preferred embodiment of the present detection method are as follows. About 2 (if hybridization is carried out in a sealed bag) to 3 (if
15 hybridization is carried out in a trough) ml of hybridization solution (5X SSPE, 0.5% SDS, and, in some instances, 1% dextran-sulfate (M.W. 500,000, although other M.W. forms would work) aids in color retention) are pre-warmed to 55 degrees C prior to use. The sample DNA is amplified by PCR using biotinylated primers, and the biotinylated product is heated to 95 degrees for 3 to 5 minutes to denature the DNA. Denaturation can
20 also be accomplished by adding 5 μ l of 5 M NaOH to 100 μ l of PCR product (final NaOH concentration is 250 mM). About 15 μ l of SA-HRP stock (20 μ g/ml, stored at 4 degrees C and never frozen) are then added to the 2 to 3 ml of hybridization solution, and then, 20 μ l of the still hot, denatured PCR product are added to the mixture. If alkali denaturation is used, then one needs to use more PCR product to maintain the same level of sensitivity
25 attained with heat denaturation. Typically 25 to 50 μ l of PCR product are used with 20 to 40 μ l of the SA-HRP stock solution. Best results are obtained when the strepavidin and the biotin are in approximate molar equivalency, i.e., about 300 ng of SA-HRP (measured in HRP) are used for every 6 pmol of biotinylated PCR product used for hybridization. The PCR product should always be added last and immediately after denaturation.

30 If the hybridization is carried out in a sealed bag, all air bubbles should be removed prior to sealing the bag. If the hybridization is carried out in a trough, the entire trough should be firmly covered with a glass plate. Hybridization is carried out for 20 minutes at 55 degrees C in a shaking water bath set at a moderate to high shaking speed, i.e., 50 to

200 rpm. The wash solution (2X SSPE, 0.1% SDS) is pre-warmed to 55 degrees during the hybridization step. After hybridization, all filters are placed in a bowl containing 200 to 300 ml of pre-warmed wash solution and washed for 8 to 10 minutes in a shaking water bath at 55 degrees C.

5 Color development is accomplished at room temperature and usually in a shaking water bath as follows if the chromogen is TMB. The filters are rinsed in 200 to 300 ml of room temperature wash solution for 5 minutes, then transferred to 200 to 300 ml of Buffer C (100 mM NaCitrate, pH = 5.0) and rinsed for 5 minutes, then incubated for 5 minutes in 40 ml of Buffer C containing 2 ml of TMB (2 mg/ml in 100% ethanol and stored at 4 degrees C), then transferred to a fresh dye solution (composed of 40 ml of Buffer C and 2 ml of TMB) containing 4 μ l of 30% hydrogen peroxide, and the color is allowed to develop 10 for 5 to 15 minutes. The color development is stopped by rinsing the filters twice with water; the filters can be dried and stored if protected from light. If the typing is weak (faint dots), the procedure is repeated using 50 μ l of the PCR product and 40 μ l of the SA-HRP during the hybridization step. If leuco dye is used in place of TMB, then one replaces the 15 Buffer C rinse with a rinse in 200 to 300 ml of 1X PBS, after which the filters are placed in 25 ml of a mixture of the dye and hydrogen peroxide (the same formulation as in Kodak Sure-Cell™ kits). The development time is 5 to 10 minutes; color development is stopped by washing the filters twice in PBS.

Example 5

20 Detection of Beta-thalassemia Mutations

Although there are over 54 characterized mutations of the beta-globin gene that can give rise to beta-thalassemia, each ethnic group in which this disease is prevalent has a limited number of common mutations (see Kazazian *et al.*, 1984, *Nature* 310:152-154; Kazazian *et al.*, 1984, *EMBO J.* 3:593-596; and Zhang *et al.*, 1988, *Hum. Genet.* 78:37- 25 40). In Mediterranean populations, eight mutations are responsible for over 90% of the beta-thalassemia alleles.

Probes were synthesized that are specific for each of these eight mutations as well as their corresponding normal sequences. The probes were given 400 nt poly-dT tails with terminal transferase and applied to membranes. Various amounts of each probe were 30 applied to twelve duplicate nylon filters, irradiated at 40 mJ/cm², hybridized with amplified beta-globin sequences in genomic DNA samples, and color developed. The result is

shown in Figure 6. In the figure, the beta-thalassemia locus that is detected by each immobilized probe pair is written at the top of the filters. For each filter, the upper row contains the probes that are specific for the normal sequence, and the lower row contains the probes specific for the mutant sequences. The beta-globin genotype of each sample is noted at the right of the corresponding filter. The name, amount applied to the membrane (in pmols and noted parenthetically), specificity, and sequence of each probe is shown below.

	<u>Probe</u>	<u>Allele Specificity</u>	<u>Sequence</u>
	RS187 (8)	Normal Beta ¹⁻¹¹⁰	5'-TAGACCAATAGGCAGAGAG
	RS188 (8)	Mutant Beta ¹⁻¹¹⁰	5'-CTCTCTGCCTATTAGTCTA
10	RS87 (4)	Normal Beta ³⁹	5'-CCTTGGACCCAGAGGTTCT
	RS89 (4)	Mutant Beta ³⁹	5'-AGAACCTCTAGGTCCAAGG
	RS189 (0.33)	Normal Beta ¹⁻⁶	5'-CTTGATACCAACCTGCCA
	RS190 (0.33)	Mutant Beta ¹⁻⁶	5'-TGGGCAGGTTGGCATCAAG
	RS191 (1)	Mutant Beta ¹⁻¹	5'-TGGGCAGATTGGTATCAAG
15	RS192 (4)	Normal Beta ²⁻¹	5'-CCATAGACTCACCCTGAAG
	RS193 (4)	Mutant Beta ²⁻¹	5'-CTTCAGGATGAGTCTATGG
	RS201 (2)	Normal Beta ²⁻⁷⁴⁵	5'-GCAGAATGGTAGCTGGATT
	RS202 (2)	Mutant Beta ²⁻⁷⁴⁵	5'-GCAGAATGGTACCTGGATT
	RS196 (4)	Normal Beta ^{6,8}	5'-ACTCCTGAGGAGAAGTCTG
20	RS197 (4)	Mutant Beta ⁶	5'-GACTCCTGGGAGAAGTCTG
	RS198 (4)	Mutant Beta ⁸	5'-TGACTCCTGAGGAGGTCTG

Because the beta-thalassemia mutations are distributed throughout the beta-globin gene, biotinylated PCR primers that amplify the entire gene in a single 1780 bp amplified product were used. The primers used for the amplification are shown below.

RS151 is 5'-ATCACTTAGACCTCACCTG

RS152 is 5'-GACCTCCCACATTCCCTTTT

This amplification product encompasses all known beta-thalassemia mutations. Following hybridization and color development, the beta-globin genotypes could be determined by noting the pattern of hybridization, as shown in Figure 6.

Unlike the DQalpha typing system, two probes are needed to analyze each mutation -- one specific for the normal sequence and one specific for the mutant sequence -- to differentiate normal/mutant heterozygous carriers from mutant/mutant homozygotes. A

complicating factor in this analysis is caused by apparent secondary structure in various portions of the relatively long beta-globin amplification product that interferes with probe hybridization. The relatively high stringency needed to minimize this secondary structure requires the use of longer (19 nt hybridizing regions) probes to capture the amplified beta-globin fragment. Because this constraint would not permit varying the length of the probes to compensate for different hybridization efficiencies, the balancing of signal intensities was accomplished by adjusting the amount of each oligonucleotide applied to the membrane.

We Claim:

1. As assay reagent comprising an oligonucleotide probe immobilized on a solid support, said probe comprising a hybridizing region that is a nucleotide sequence complementary to a specific nucleotide sequence to be detected covalently attached to a spacer arm which is longer than said hybridizing region, wherein said probe is immobilized on said solid support by a covalent bond between said solid support and said spacer arm, such that the hybridizing region of said probe can hybridize to said nucleotide sequence to be detected under hybridizing conditions.
2. The assay reagent of Claim 1, wherein said spacer arm is a polynucleotide tail, said solid support contains primary or secondary amines prior to attachment of said probe to said support, and said attachment is formed by ultraviolet light irradiation of said probe on said support.
3. The assay reagent of Claim 2, wherein said probe is an oligodeoxyribonucleotide.
4. The assay reagent of Claim 3, wherein said tail contains from 200 to 800 nucleotides.
5. The assay reagent of Claim 4, wherein said support comprises nylon.
6. The assay reagent of Claim 4, wherein said tail comprises at least 150 pyrimidine nucleotides.
7. The assay reagent of Claim 6, wherein said pyrimidine nucleotides are thymidine nucleotides.
8. The assay reagent of Claim 6, wherein said hybridizing region is a sequence of nucleotides from 17 to 23 nucleotides in length.

5 9. The assay reagent of Claim 1 that comprises a set of probes immobilized on a solid support, wherein said set of probes comprises two or more members, each member of said set having a hybridization region different from every other member of said set, wherein each member is immobilized on said solid support at a discrete location separate from every other probe of said set.

 10. The assay reagent of Claim 9, wherein each probe of said set of probes is an oligodeoxyribonucleotide.

 11. The assay reagent of Claim 9, wherein one member probe of said set serves as a positive control.

10 12. The assay reagent of Claim 9, wherein said member probes of said set are complementary to nucleic acid sequences of microorganisms.

 13. The assay reagent of Claim 9, wherein said member probes of said set are complementary to variant alleles of a genetic locus.

 14. The assay reagent of Claim 13, wherein said genetic locus is an HLA locus.

15 15. The assay reagent of Claim 14, wherein said HLA locus is DQalpha.

 16. The assay reagent of Claim 9, wherein said spacer arm is a polynucleotide tail, and said solid support contains primary or secondary amines prior to attachment of said probe to said support, and said attachment is formed by ultraviolet light irradiation of said probe on said support.

20 17. A method for preparing the assay reagent of Claim 2, which method comprises: (a) contacting said probe with a solid support comprising amine groups; and (b) irradiating the support prepared in step (a) with ultraviolet light.

18. A method for detecting the presence of a nucleic acid sequence in a sample, which method comprises: (a) contacting said sample with the assay reagent of Claim 1 under conditions that allow for hybridization of complementary nucleic acid sequences; and (b) determining if hybridization has occurred.

5 19. A kit comprising the assay reagent of Claim 1 and instructions for detecting specific nucleic acids with said reagent.

10 20. An assay reagent comprising a set of oligonucleotide probes covalently attached to a solid support, wherein said set of probes comprises two or more members, each member of said set having a hybridization region different from every other member of said set, wherein each member is immobilized on said support at a discrete location separate from every other probe of said set.

 21. The reagent of Claim 20, also comprising a labeled polynucleotide hybridized to one of said probes, wherein said polynucleotide comprises at least 50 nucleotides.

15 22. The reagent of Claim 20, also comprising a target sequence from a sample hybridized to an immobilized probe of said set and a colored or fluorescent compound immobilized on said support at the location of said hybridized probe.

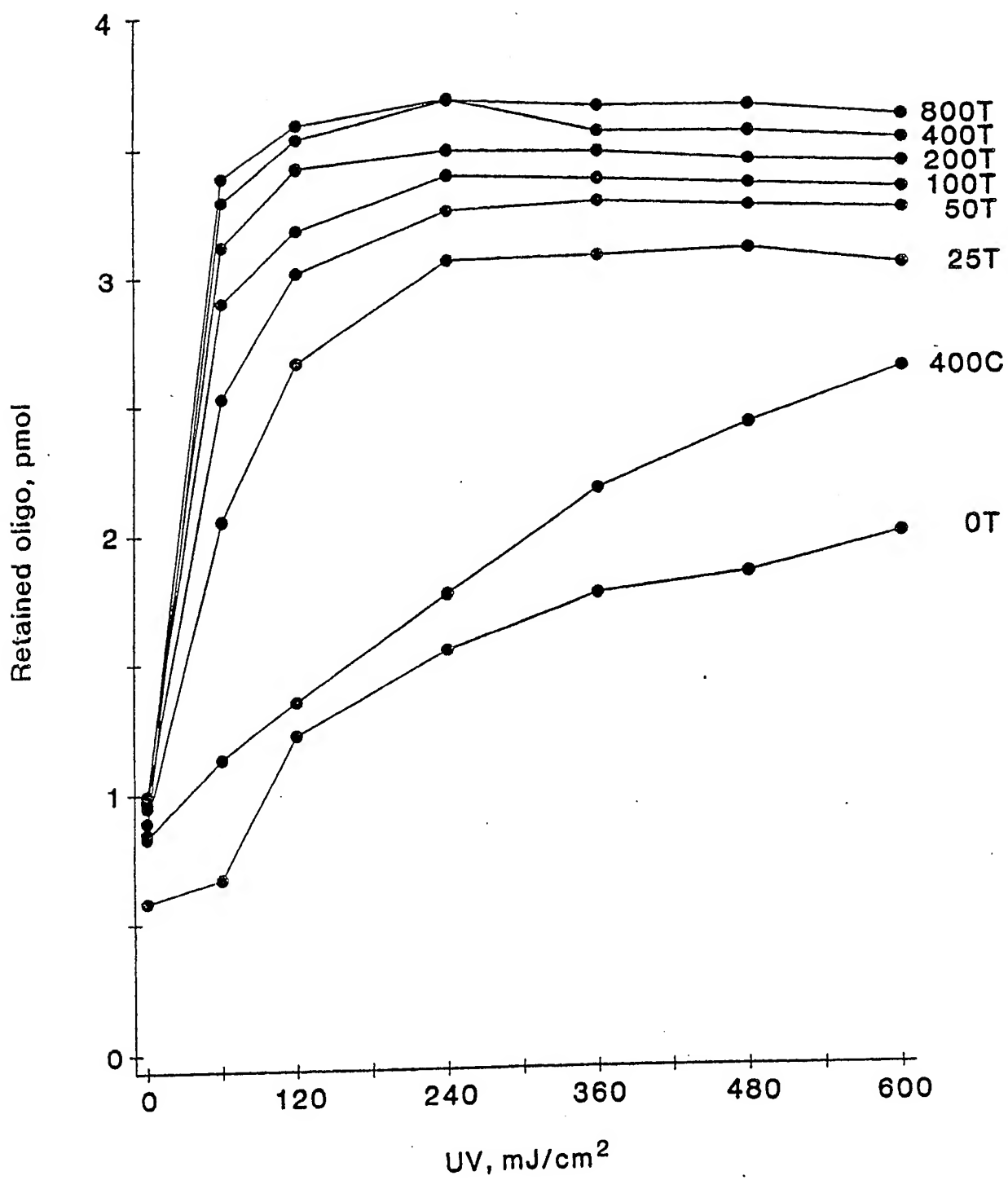


FIG. 1

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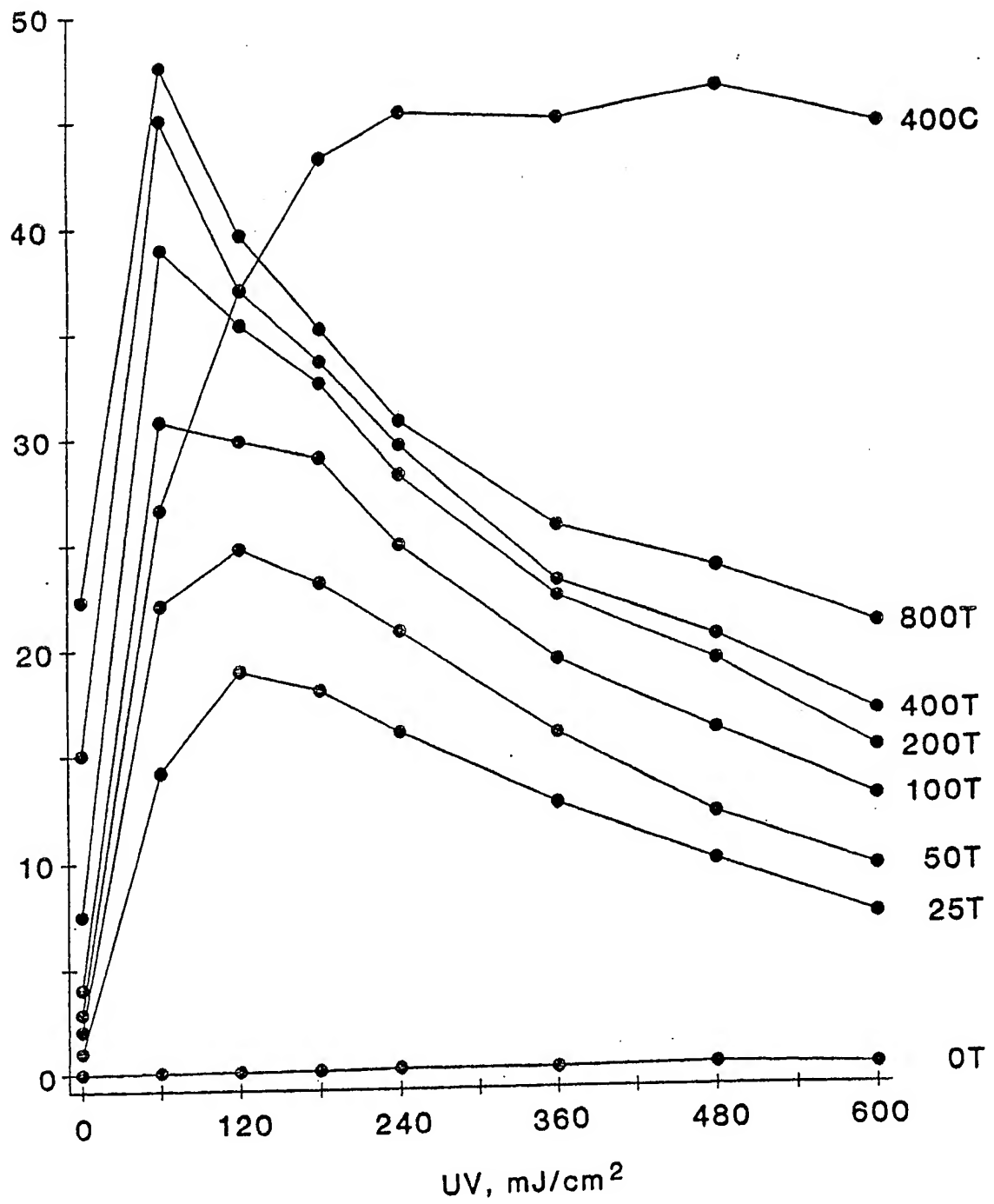


FIG. 2

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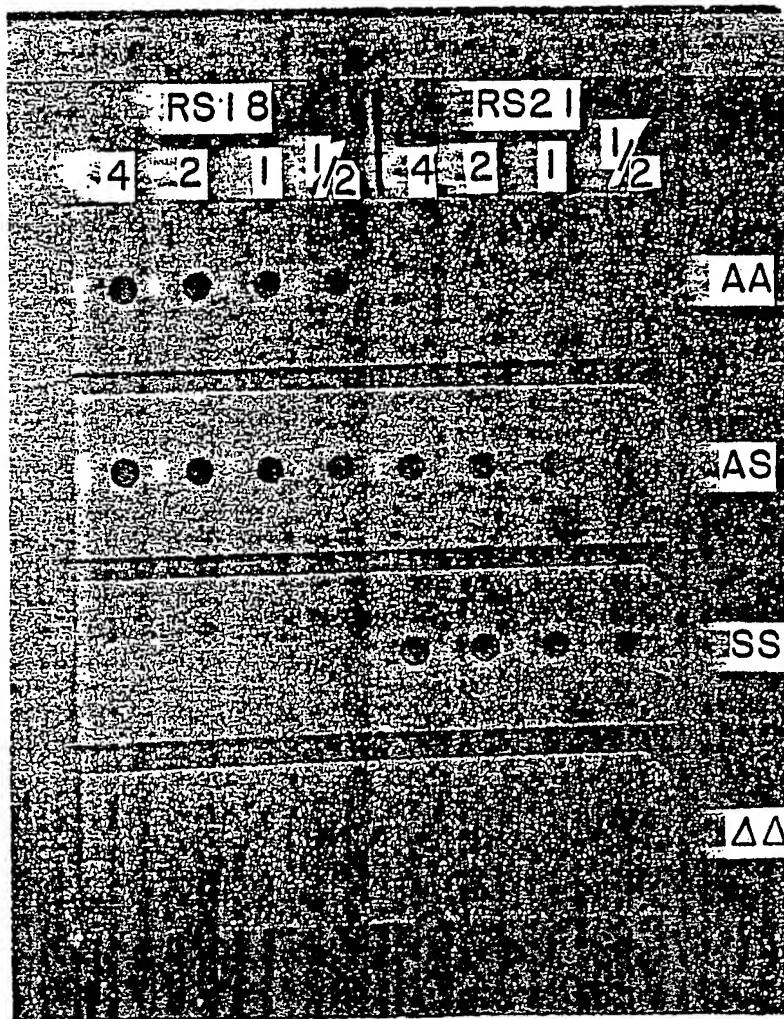


FIG. 3

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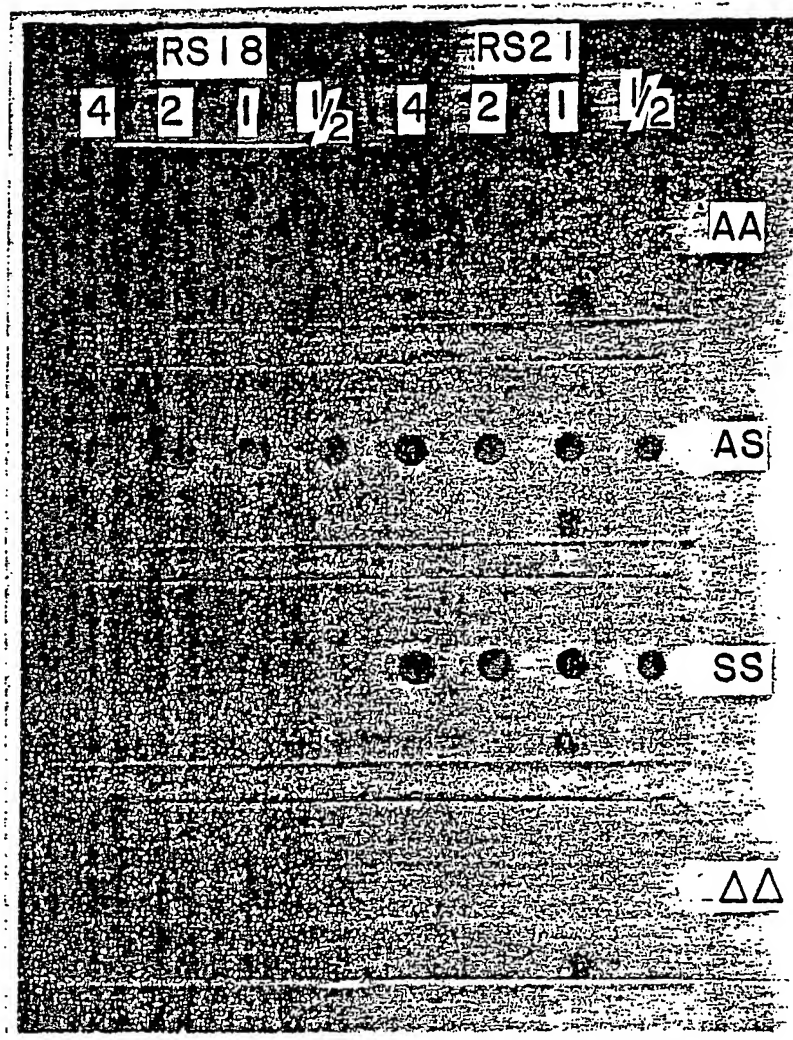


FIG. 4

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All	DQA 1	DQA 2	DQA 3	DQA 4	DQA 1.1	DQA 1.2, 1.3, 4	DQA 1.3	not DQA 1.3	
●	●				●			●	DQA 1.1/1.1
●	●					●		●	DQA 1.2/1.2
●	●					●	●		DQA 1.3/1.3
●		●						●	DQA 2/2
●			●					●	DQA 3/3
●				●		●			DQA 4/4
●									DQA 2/4
●						●			DQA 1.2/4
●								●	DQA 1.2/3
●		●		●		●		●	DQA 2/4
●	●	●			●			●	DQA 1.1/2
●			●	●		●		●	DQA 3/4

FIG. 5

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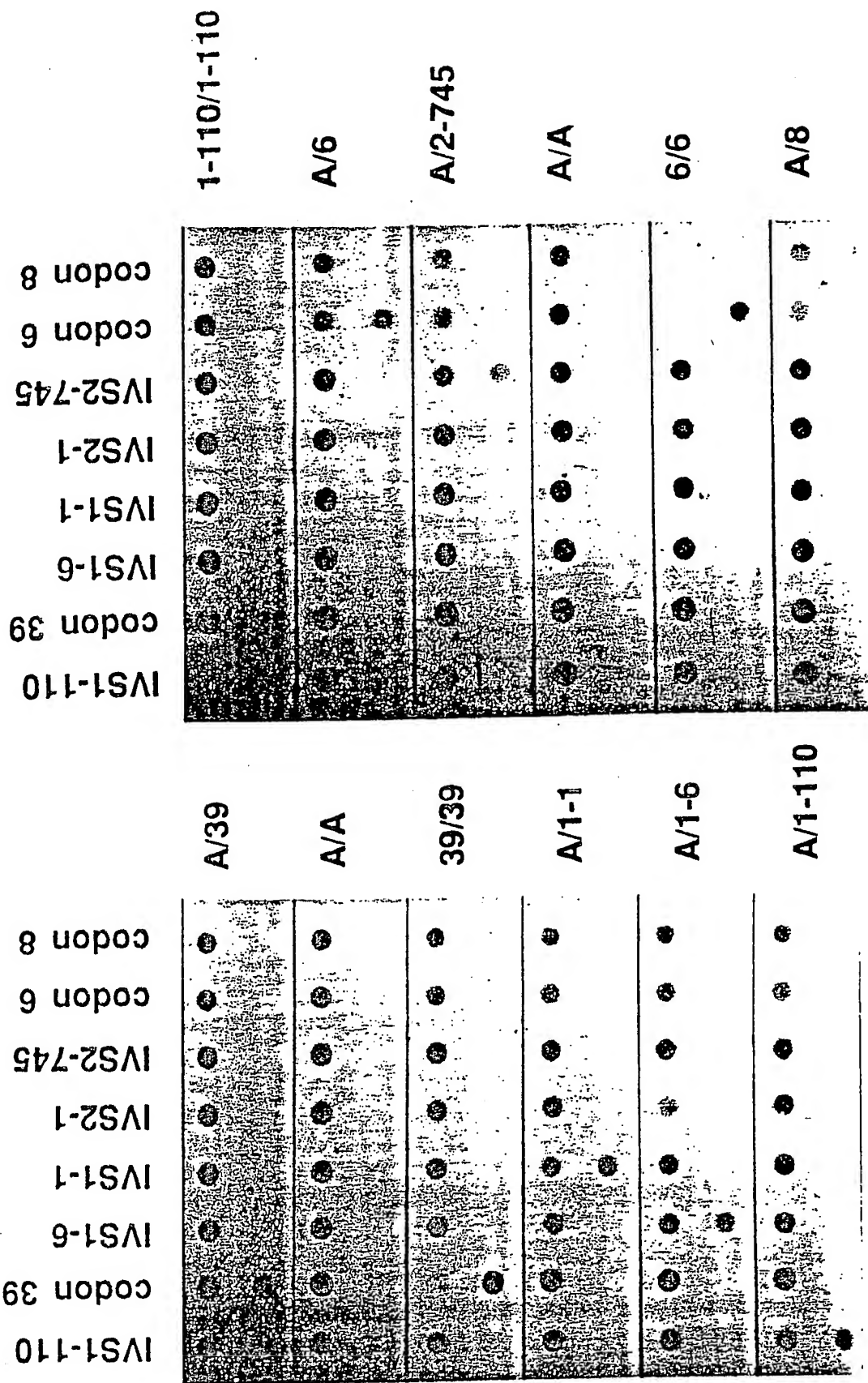


FIG. 6

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 89/02170

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
IPC ⁴ : C 12 Q 1/68		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
IPC ⁴	C 12 Q 1	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	EP, A, 0130523 (MOLECULAR DIAGNOSTICS INC.) 9 January 1985, see page 2, lines 2-33; page 3, lines 17-31; page 13, lines 32-34; page 14, lines 29-30; page 16, line 27 - page 17, line 2	1-4,6,8,10,13,14,16-19,22
Y	--	1-22
X	Proceedings of the National Academy of Sciences USA, vol. 81, April 1984, (Washington, D.C., US), G.M. Church et al.: "Genomic sequencing", pages 1991-1995, see the whole article cited in the application	5,13,14
Y	cited in the application --	1-22
X	Tetrahedron Letters, vol. 22, no. 34, 1981, Pergamon Press Ltd (GB), I. Saito et al.: "Photochemical ring opening of thymidine and thymine in ./.:	6,7
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>¹⁰ Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report	
27th September 1989		
International Searching Authority	Signature of Authorized Officer	
EUROPEAN PATENT OFFICE	T.K. WILLIS	

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	the presence of primary amines", pages 3265-3268, see the whole article cited in the application --	
Y	EP, A, 0142299 (FUJIREBIO INC.) 22 May 1985, see page 3, lines 24-28; page 2, lines 22-37 --	1-21
Y	Nucleic Acids Research, vol. 14, no. 24, 1986, IRL Press Ltd (Oxford, GB), H.B. Gamper et al.: "Reverse southern hybridization", pages 9943-9954, see the whole article ----	1-21

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 8902170
SA 29159

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 18/10/89. The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0130523	09-01-85	US-A- 4542102	17-09-85
		US-A- 4713326	15-12-87
		AU-B- 563558	16-07-87
		AU-A- 3025684	10-01-85
		CA-A- 1215703	23-12-86
		DE-A- 3471689	07-07-88
		JP-A- 60036496	25-02-85

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